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GENETIC TOXICITY ASSESSMENT OF 1,3-DICHLOROTETRAFLUOROBENZENE

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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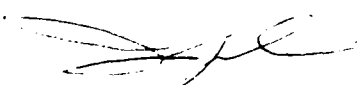

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1,3-Dichlorotetrafluorobenzene (DCFB), a fully substituted halogenated benzene, was evaluated using *in vitro* bioassays to assess its potential genotoxic activity. The assays conducted were the Ames salmonella/microsomal mutagenicity assay, the Chinese hamster ovary (CHO) gene mutation assay, the CHO/sister chromatid exchange (SCE) and chromosome aberration assay, the BALB/c-3T3 cell transformation assay and an *in vivo/in vitro* unscheduled DNA synthesis assay. DCFB did not demonstrate genotoxic potential in any of the assays. Thus the results of the test battery would predict no genetic risk from exposure to DCFB.

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PREFACE

The research reported in this document was conducted by Hazleton Laboratories America, Inc. under a subcontract to NSI Technology Services Corporation in support of the Toxic Hazards Research Unit (THRU). The THRU is the contractor-operated effort of the Toxic Hazards Division of the Harry G. Armstrong Aerospace Medical Research Laboratory located at Wright-Patterson Air Force Base, OH. During the initiation and conduct of these studies Melvin E. Andersen, Ph.D; Lt Col Harvey J. Clewell, III; and Lt Col Michael B. Ballinger served consecutively as the contract technical monitor

The experimental work reported here was begun on 24 January 1989 and completed 16 September 1989. The genotoxicity assays were conducted at Hazleton Laboratories America facilities in Kensington, MD. The results of their work were reported to NSI in separate reports on each assay. These separate reports were edited by NSI and organized such that the results of each assay are provided in a separate paper. Each paper is authored by the investigator that conducted the study and a final discussion paper is provided to collectively consider the results from the individual studies. The final, unabridged reports received from Hazleton Laboratories with copies of the raw data, Quality Assurance Statements, and Good Laboratory Practice Compliance and Certification Statements for each of the studies will be archived in the Quality Assurance Archives of the THRU.

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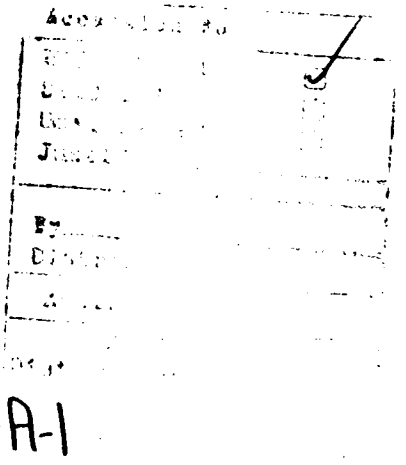

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INTRODUCTION

Kutzman, R. S.

A fully substituted halogenated benzene, 1,3-dichlorotetrafluorobenzene (DCFB), is being considered as a test agent to assess the effectiveness of chemical defense procedures and equipment. It is anticipated that when used in training exercises the breath of trainees will be analyzed, for DCFB, following the exercises to estimate what their dose of a chemical agent would have been. To more fully evaluate the safety of these proposed training agents, *in vitro* and *in vivo/in vitro* tests were conducted to determine their genotoxic potential.

The skin sensitization and acute inhalation toxicity potential of a structurally similar compound, chloropentafluorobenzene (CPFB), was investigated and found to be unremarkable. However, it did produce mild skin and conjunctival irritation in treated rabbits (Kinkead, et al., 1987). Repeated inhalation exposures of Fischer 344 rats and B6C3F1 mice to 0.25, 0.80, or 2.50 mg CPFB/L of air for 6 h/day, 5 days/week for 3 weeks resulted in a reduced rate in the growth of the rats but not of the mice (Kinkead, et al., 1989). Following these exposures both species demonstrated increased liver weights. Histologically the tissue exhibited mild hepatocytomegaly with increased granular eosinophilic cytoplasm. Bone marrow samples from the exposed mice were evaluated for evidence of CPFB induced genetic changes; all findings were negative.

Several studies have been conducted to assess the genotoxic potential of CPFB (Tu et al., 1986; Steele, 1987) and the results have been equivocal. Therefore, a battery of genotoxicity assays was designed to assess the potential of DCFB to induce genetic damage and to compare its genotoxic activity to that of CPFB in the same series of assays (Kutzman, et al., 1990).

This report is arranged such that the results of each assay in the battery are provided in a separate paper. Each paper is authored by the investigator who conducted the study. A final discussion paper is provided to collectively consider the results from the individual studies. In addition, the report appendices (Appendix A and B) provide information on the efforts to conduct *in vitro* unscheduled DNA synthesis assays in closed glass culture vessels. It was determined that acceptable culture conditions for rat hepatocytes with such vessel demands would require research beyond that needed to address the biological activity of DCFB. The initial work has been included in this report to provide background for investigators who may pursue the development of the appropriate culture conditions.

REFERENCES

Kinkead, E.R., W.J. Bashe, D.M. Brown, and S.S. Henry (1987) Evaluation of the inhalation toxicity and sensitization potential of chloropentafluorobenzene. In: (W.E. Houston, R.S. Kutzman, R. L. Carpenter, eds.) *1986 Toxic Hazards Research Unit Annual Report*. pp. 131-135. AAMRL-TR-87-020, Harry G. Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH; NMRI-87-2, Naval Medical Research Institute, Bethesda, MD.

Kinkead, E.R., B.T.Culpepper, H.G. Wall, R.S. Kutzman, C.D. Flemming, C.J. Hixson, and R.R. Tice (1989). Evaluation of the Potential of Inhaled Chloropentafluorobenzene to Induce Toxicity in F-344 Rats and B6C3F1 Mice and Sister Chromatid Exchanges and Micronuclei Formation in B6C3F1 Mice. AAMRL-TR-89-037 Harry G. Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH.

Kutzman, R.S, B.C. Myhr, T.E. Lawlor, D.C. Valentine, R.R. Young, H. Murli, and M.A. Cifone (1990). Genetic Toxicity Assessment of Chloropentafluorobenzene. AAMRL-TR-90-048, Harry G. Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH.

Steele, V. (1987). Biological Activity of Chloropentafluorobenzene. AAMRL-TR-87-039, Harry G. Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH.

Tu, A., M.G. Broome, and A. Sivak (1986). Evaluation of Chloropentafluorobenzene in a Battery of *In Vitro* Short Term Assays. AAMRL-TR-86-003 Harry G. Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH.

SECTION 2

MUTAGENICITY TESTING OF 1,3-DICHLOROTETRAFLUOROBENZENE IN THE *SALMONELLA*/REVERSE MUTATION ASSAY (AMES TEST) PREINCUBATION METHOD

Lawlor, T.E.[a] and Valentine D.C.[a]

INTRODUCTION

The mutagenic activity of 1,3-dichlorotetrafluorobenzene (DCFB) was examined in the *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor-induced rat liver.

The *Salmonella*/Mammalian-microsome reverse mutation assay (Ames Test) detects point mutations, both frameshifts and/or base pair substitutions, in bacteria. The strains of *Salmonella typhimurium* used in this assay are histidine auxotrophs by virtue of conditionally lethal mutations in their histidine operon. When these histidine-dependent cells (*his*-) are exposed to the test article and grown under selective conditions (minimal media with a trace amount of histidine) only those cells which revert to histidine independence (*his* +) are able to form colonies. The trace amount of histidine in the media allows all the plated bacteria to undergo a few cell divisions: this growth is essential for mutagenesis to be fully expressed. The *his* + revertants are readily discernable as colonies against the limited background growth of the *his*- cells. By utilizing several different tester strains, both base pair substitution mutations and frameshift mutations can be detected. The Ames Test has been shown to be a sensitive, rapid and accurate indicator of the mutagenic activity of many materials including a wide range of chemical classes.

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The *rfa* wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall. The second

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mutation, a deletion of the *uvr*B gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototropy) by frameshift mutagens. TA1535 is reverted by base substitution mutagens and TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strain TA100 and 10 doses of test article ranging from 10,000 to 10.0 µg per plate, one plate per dose, both in the presence and absence of microsomal enzymes.

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames et al. (1975) and Yahagi et al. (1975).

The test article, DCFB (Lot No. 01609LV), a clear, colorless liquid, was stored at room temperature.

A 10% F-68 Pluronic® solution (w/v in deionized water, sterilized with a 0.45 µm filter) was used as the vehicle and the test article formed a suspension at 200 mg per mL which was the most concentrated stock dilution of test article prepared. This stock suspension was mixed with a tissuemizer for approximately one minute to enhance the homogeneity of the suspension.

Media and Reagents

Top Agar for Selection of Histidine Revertants: Minimal top agar was prepared with 0.7% agar (w/v) and 0.5% NaCl (w/v). After sterilization by autoclaving, the molten top agar was distributed into sterile bottles and stored at room temperature. Immediately before its use in the mutagenicity assay, the top agar was melted and supplemented with 10 mL per 100 mL agar of a sterile solution that contained 0.5 mM L-histidine and 0.5 mM D-biotin.

Minimal Bottom Agar: Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956).

Nutrient Broth: Nutrient Broth used for growing overnight cultures of the tester strains was Vogel-Bonner salt solution supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Nutrient Bottom Agar: Nutrient bottom agar (for tester strain culture density determination) was Vogel-Bonner minimal medium E supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Exogenous Metabolic Activation

Liver Microsomal Enzymes - S9 Homogenate: S9 Liver homogenate for use in the mutagenicity assay, prepared as described below, was purchased from Molecular Toxicology, Inc., College Park, MD, 20742, Lot # 0264, 39.8 mg of protein per mL.

Species, Strain, Sex, Inducer: Liver microsomal enzymes were prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 (200 mg per mL in corn oil) at 500 mg/kg. Five days after i.p. injection with the Aroclor, the rats were sacrificed by decapitation, and their livers were excised.

Homogenate Preparation: The preparation of the microsomal enzyme fraction was carried out with sterile glassware and solutions at 0-4°C. The livers were excised and placed in a beaker containing three volumes of 0.15M KCl (3 mL/g of wet liver) and homogenized. The homogenate was centrifuged at 9000 × g for 10 min. Small volumes of the supernatant (referred to by Ames as the S9 fraction) were distributed into freezing ampules which were stored at ≤ -70°C.

S9 Characterization: The S9 homogenate was characterized (using the Ames Assay) for its ability to metabolize selected promutagens to their mutagenic forms, as described by deSerres and Shelby (1979).

S9 Mix: The S9 mix was prepared immediately before its use in the mutagenicity assay. One mL of the microsomal enzyme reaction mixture (S9 mix) contained the following components:

H20	0.70 mL
1.00M NaH2PO4/Na2HPO4, pH 7.4	0.10 mL
0.25M Glucose-6-phosphate	0.02 mL
0.10M NADP	0.04 mL
0.2M MgCl2/0.825M KCl	0.04 mL
S9 Homogenate	0.10 mL
	1.00mL

When S9 was required, 0.5 mL of the S9 mix was added to the preincubation mixture.

Sham S9 Mix: The Sham S9 mix was prepared immediately before its use in the mutagenicity assay. One mL of the Sham S9 mix contained the following components:

H2O	0.90 mL
1.00M NaH2PO4/Na2HPO4, pH 7.4	0.10 mL
	1.00 mL

When S9 was not required, 0.5 mL of the Sham S9 mix was added to the preincubation mixture, in place of the S9 mix.

Test System

Tester Strains: The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538. The description of the tester strains that follows is a summarization of the description provided by Ames et al. (1975).

TESTER STRAIN GENOTYPES

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R Factor
TA1535	TA1537	TA1538	*rfa*	*uvr*B	-
TA100		TA98	*rfa*	*uvr*B	+ R

Source of Tester Strains: The tester strains were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

Storage of the Tester Strains

Frozen Permanent Stocks: Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 mL/mL of culture) and freezing small aliquots (approximately 1.5 mL) at ≤ -70°C.

Master Plates: Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 µM), biotin (3 µM), and for strains containing the R-factor, ampicillin (25 µg/mL). Tester strain master plates were stored at approximately 4°C.

Preparation of Overnight Cultures: Overnight cultures were prepared by transferring a colony from the appropriate master plate to a flask containing culture medium. In order to assure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring. Inoculated flasks were placed in a shaker/incubator which was programed to begin operation (shaking, 125 ± 12.5 rpm; incubation, 37 ± 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance

(%T) reading on a spectrophotometer. Overgrowth of cultures can result in their loss of sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached.

Confirmation of Tester Strain Genotypes

Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay.

rfa Wall Mutation: The presence of the *rfa* wall mutation was confirmed by demonstration of sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

pKM101 Plasmid R-factor: The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. A sample of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

Characteristic Number of Spontaneous Revertants: The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 µl aliquots of the culture along with the appropriate vehicle on selective media.

Experimental Design

The tester strains used in this study were TA98, TA100, TA1535, TA1537 and TA1538. The assay was conducted using three plates per dose in the presence and absence of microsomal enzymes. Seven doses of the test article were tested, from 5,000 to 5.00 µg per plate in the presence of S9 and from 2,500 to 1.00 µg per plate in the absence of S9 (It should be noted that in this report, the doses have been expressed as µg of test article per plate. This reflects the fact that the exposure of the test system to the test article does not cease at the end of the 20 min preincubation period. A dose of 10,000 µg per plate indicates that the bacteria are exposed to a concentration of 15,400 µg of test article per mL of preincubation mixture for 20 min prior to being combined with 2 mL of overlay agar and being overlaid onto 25 mL of bottom agar).

Mutagenicity Assay: The mutagenicity assay was performed using tester strains TA98, TA100, TA1535, TA1537 and TA1538, both in the presence and absence of microsomal enzymes (S9 mix). Seven doses of the test article were tested along with the appropriate vehicle and positive controls. The doses tested were selected based on the results of the dose rangefinding study.

Frequency and Route of Administration: The test system was exposed to the test article via the preincubation modification of the Ames Test originally described by Yahagi et al. (1975). This methodology has been shown to detect mutagenicity with certain classes of chemicals, such as nitrosamines or volatile compounds which may not be detected in the standard plate incorporation method. All doses of test article, vehicle controls and positive controls were preincubated and plated in triplicate.

Dose Rangefinding Study: The dose rangefinding study was performed using tester strain TA100 both in the presence and absence of microsomal enzymes. Ten doses of test article were tested (one plate per dose). The dose rangefinding study was performed using the same methodology as was used for the mutagenicity assay. Cytotoxicity in this study is detectable as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Routinely, the maximum dose selected to be tested in the mutagenicity assay should demonstrate cytotoxicity if possible.

The growth inhibitory effect (cytotoxicity) of the test article on tester strain TA100 is generally representative of that observed on the other tester strains and because of TA100's comparatively high number of spontaneous revertants per plate, gradations of cytotoxicity can be readily discerned from routine experimental variation. Also, the cytotoxicity induced by a test article in the presence of microsomal enzymes may vary greatly from that observed in the absence of microsomal enzymes. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the microsomal enzymes.

Controls

Positive Controls: All combinations of positive controls and tester strains plated concurrently with the assay are listed below.

POSITIVE CONTROL AND TESTER STRAIN COMBINATIONS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 µg
TA98	–	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	–	sodium azide	2.0 µg
TA1535	+	2-aminoanthracene	2.5 µg
TA1535	–	sodium azide	2.0 µg
TA1537	+	2-aminoanthracene	2.5 µg
TA1537	–	ICR-191	2.0 µg
TA1538	+	2-aminoanthracene	2.5 µg
TA1538	–	2-nitrofluorene	1.0 µg

Source and Grade of Positive Control Articles:

2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade
2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98%
sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade
ICR-191 (CAS #1707-45-0), Polysciences Inc, >95% pure.

Vehicle Controls: Appropriate vehicle controls were plated for all tester strains both in the presence and absence of S9. The vehicle control was plated, using an aliquot of vehicle equal to the aliquot of test article dilution plated, along with an aliquot of the appropriate tester strain, on selective agar.

Sterility Determinations: To determine the sterility of the test article, the highest test article dose used in the mutagenicity assay was checked for sterility by plating a volume equal to that used in the assay on selective agar. To determine the sterility of the S9 mix, 0.5 mL were plated on selective agar.

Plating Procedures

The plating procedures employed are similar to those described by Ames et al. (1975) and Yahagi et al. (1975). These procedures were employed for both the Dose Rangefinding Study and the Mutagenicity Assay.

Test System Identification: Each plate was labeled with a code system which identified the test article, tester strain, test phase, dose, and activation condition.

Test Article Plating Procedure: The test article was diluted and the S9 mix was prepared immediately before their use in any experimental procedure.

When S9 mix was required, 0.5 mL of S9 mix was added to 13 x 100 mm glass, screw-cap, culture tubes, pre-heated to 37 ± 2°C. To these tubes were added 100 µL of appropriate tester strain and 50 µL of vehicle or test article dilution. When S9 mix was not required, 0.5 mL of Sham S9 (0.1M phosphate buffer) was substituted for the S9 mix. Once all components had been added to a tube, it was tightly capped, and after vortexing, the mixture was allowed to incubate for 20 ± 2 min at 37 ± 2°C. The caps were then removed and 2.0 mL of molten selective top agar was added to each tube and, after vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for approximately 48 h at 37 ± 2°C.

Scoring Plates

Plates that were not scored immediately after the 48 ± 4.8 h incubation period were held at 4 ± 2°C until such time that scoring could occur.

Evaluation of the Bacterial Background Lawn: The condition of the background bacterial lawn was evaluated for evidence of cytotoxicity due to the test article by using a dissecting microscope. The cytotoxicity was scored relative to the vehicle control plate. In addition to the cytotoxicity, any test article precipitate observed on the plates is also noted at the appropriate dose on the data tables.

Colony Counting: Revertant colonies for a given tester strain and activation condition were counted either entirely by automated colony counter or entirely by hand. If the plates contained sufficient test article precipitate to interfere with automated colony counting, then they were counted manually.

Analysis of the Data: For all replicate platings, the mean number of revertants per plate was calculated and the standard deviation around the mean was also calculated.

Criteria for Determination of a Valid Test

The following criteria must be met for the assay to be considered valid.

rfa Wall Mutation: To demonstrate the presence of the deep rough mutation, all tester strain cultures must exhibit sensitivity to crystal violet.

pKM101 Plasmid R-Factor: To demonstrate the presence of the pKM101 Plasmid R-factor, all tester strains must exhibit resistance to ampicillin.

Characteristic Number of Spontaneous Revertants: All tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The acceptable ranges are as follows.

TA98	10	-	60
TA100	80	-	240
TA1535	5	-	45
TA1537	3	-	21
TA1538	5	-	35

Tester Strain Culture Density: To ensure that appropriate numbers of bacteria are plated, tester strain culture density must be greater than or equal to 5.0×10^8 bacteria per mL.

Positive Control Values: All positive controls must exhibit at least a three-fold increase in the number of revertants per plate over the mean value for the vehicle control for the respective strain.

Cytotoxicity: A minimum of three non-toxic doses are required to evaluate assay data.

Evaluation of Test Results

Tester Strains TA98 and TA100: For a test article to be considered positive, it must cause at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

Tester Strains TA1535, TA1537 and TA1538: For a test article to be considered positive, it must cause at least a 3-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

RESULTS AND DISCUSSION

Dose Rangefinding Study

Doses of DCFB to be tested in the mutagenicity assay were selected based on the results of the dose rangefinding study conducted using tester strain TA100 in both the presence and absence of S9 (one plate per dose). Ten doses of test article, from 10,000 to 10.0 µg were tested and the results are presented in Table 2-1. In the presence of S9, a slight thinning of the bacterial background lawn was observed beginning with the 333 µg per plate dose and a reduction in revertants was observed beginning with the 3330 µg per plate dose. In the absence of S9, a slight thinning of the background lawn was observed beginning with the 66.7 µg per plate dose and a reduction in revertants was observed beginning with the 3330 µg per plate dose.

Mutation Assay

The results of the dose rangefinding study were used to select seven doses to be tested in the mutagenicity assay. Routinely, only six doses would be tested, however, since background lawn toxicity was observed in the absence of revertant toxicity, an additional dose was tested to allow demonstration of revertant toxicity in the mutagenicity assay. The doses selected for the mutation assay ranged from 5,000 to 5.00 µg per plate in the presence of S9 and from 2,500 to 1.00 µg per plate in the absence of S9.

The mutagenicity assays for DCFB all data were acceptable and all criteria for a valid study were met. There were no positive increases in the mean number of revertants per plate with any of the tester strains either in the presence or absence of S9 (Appendix 2-B).

CONCLUSION

The results of the *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method, indicate that under the conditions of this study, DCFB did not cause a positive increase in the number

of histidine revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver

REFERENCES

Ames, B.N., J. McCann and E. Yam- -ki. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutation Research* 31:347-364.

deSerres, F.J., and M.D. Shelby. 1979. The *Salmonella*/mutagenicity assay: Recommendations. *Science* 203:563-565.

Vogel, H.J., and D.M. Bonner. 1956. Acetylornithinase of *E. coli*: partial purification and some properties, *J. Biol. Chem.* 218:97-106.

Yahagi, T., M. Degawa, Y. Seino, T. Matsushima, M. Nagao, T. Sugimura, and Y. Hashimoto. 1975. Mutagenicity of carcinogenic azo dyes and their derivatives. *Cancer Letters* 1:91-96.

TABLE 2-1. DOSE RANGEFINDING STUDY FOR DCFB

µg/PLATE	TA100 REVERTANTS PER PLATE			
	WITH S9		WITHOUT S9	
	NUMBER OF COLONIES/PLATE	APPEARANCE OF BACKGROUND LAWN*	NUMBER OF COLONIES/PLATE	APPEARANCE OF BACKGROUND LAWN*
0.00 (Vehicle) (50.0 µL)	131	1	108	1
DCFB				
10.0	123	1	109	1
33.3	133	1	80	1
66.7	126	1	75	2
100	118	1	96	2
333	86	2	63	2
667	104	3	86	2
1000	98	3	58	3
3333	21	4	0	5
6670	37	4	13	4
10000	0	5	0	5

*Background Lawn Evaluation Codes (See Appendix 2-A):
1 = normal
2 = slightly reduced
3 = moderately reduced
sp = slight precipitate
mp = moderate precipitate (requires hand count)
4 = extremely reduced
5 = absent
6 = obscured by precipitate
hp = heavy precipitate (requires hand count)

APPENDIX 2-A

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS OF BACKGROUND LAWN
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

APPENDIX 2-B
SALMONELLA MUTAGENICITY ASSAY RESULTS FOR DCFB
INDIVIDUAL PLATE COUNTS

Article ID: 1,3 Dichlorotetrafluorobenzene
Vehicle: 10% Pluronic (w/v in H_2O)
Plating Aliquot: 50.0 µL

		Revertants Per Plate															
		TA1535			TA1537[a]			TA1538			TA98[a]			TA100			Back-ground
	Dose/Plate	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	Lawn*
Microsomes: None																	
Vehicle Control		10	8	8	5	5	3	4	8	8	24	20	20	123	100	116	1
Test Article	1.00 µg	10	7	9	2	8	8	7	12	10	19	19	29	88	129	93	1
	5.00 µg	10	12	9	3	7	5	2	6	5	17	17	17	117	96	129	1
	10.0 µg	10	7	12	5	3	4	4	8	8	15	18	18	116	108	98	1
	50.0 µg	10	10	7	2	2	2	4	3	3	13	17	11	86	71	97	2
	100 µg	9	6	8	6	3	5	2	2	5	10	11	10	66	62	75	2
	625 µg	5	10	11	3	3	1	0	5	3	8	6	6	90	79	76	3
	2500 µg	10	3	2	0	0	0	0	0	0	0	0	0	0	0	39	4
Positive Control**		439	396	419	1408	1480	1525	260	304	257	152	154	138	435	413	463	1
Microsomes: Rat Liver																	
Vehicle Control		12	10	10	8	8	15	19	14	10	23	26	24	155	170	133	1
Test Article	5.00 µg	19	11	17	7	6	5	19	18	25	32	35	36	145	138	151	1
	10.0 µg	16	11	8	7	10	11	12	18	19	33	29	23	141	127	149	1
	50.0 µg	11	9	8	13	9	8	15	12	13	34	34	34	158	157	150	1
	100 µg	19	15	12	4	6	10	20	17	25	39	35	43	160	162	132	1
	500 µg	14	9	8	7	9	11	13	22	15	15	28	17	110	123	98	2
	1000 µg	16	11	17	5	9	10	14	11	14	16	13	17	123	97	104	3
	5000 µg	0	5	10	0	0	0	0	0	0	0	0	0	0	68	68	4
Positive Control***		78	100	91	102	86	106	1144	1044	910	849	760	1011	885	818	698	1

** TA1535 sodium azide @ 10 µg/plate
TA1537 quinacrine mustard @ 5 µg/plate
TA1538 2-nitrofluorene @ 10 µg/plate
TA98 2-nitrofluorene @ 10 µg/plate
TA100 sodium azide @ 10 µg/plate

*** TA1535 2-aminoanthracene @ 2.5 µg/plate
TA1537 2-aminoanthracene @ 2.5 µg/plate
TA1538 2-aminoanthracene @ 2.5 µg/plate
TA98 2-aminoanthracene @ 2.5 µg/plate
TA100 2-aminoanthracene @ 2.5 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

[a] The density of the cultures of tester strains TA1537 (0.3×10^9) and TA98 (0.3×10^9) were below the acceptable range ($\geq 0.5 \times 10^9$) However, since all other tester strain characterization indicators were acceptable (culture turbidity, ampicillin and crystal violet sensitivities, and positive and vehicle control values), the data generated with these cultures of TA1537 and TA98 have been accepted

APPENDIX 2-B (Continued)
SALMONELLA MUTAGENICITY ASSAY RESULTS FOR DCFB
INDIVIDUAL PLATE COUNTS

Article ID: 1,3 Dichlorotetrafluorobenzene
Vehicle: 10% Pluronic (w/v in H_2O)
Plating Aliquot: 50.0 µL

		Mean Revertants Per Plate with Standard Deviations										
		TA1535		TA1537[a]		TA1538		TA98		TA100		Background
	Dose/Plate	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Lawn*
Microsomes: None												
Vehicle Control		9	1	4	1	7	2	21	2	113	12	1
Test Article	1.00 µg	9	2	6	3	10	3	22	6	103	22	1
	5.00 µg	10	2	5	2	4	2	17	0	114	17	1
	10.0 µg	10	3	4	1	7	2	17	2	107	9	1
	50.0 µg	9	2	2	0	3	1	14	3	85	13	2
	100 µg	8	2	5	2	3	2	10	1	68	7	2
	625 µg	9	3	2	1	3	3	7	1	82	7	3
	2500 µg	5	4	0	0	0	0	0	0	13	23	4
Positive Control**		418	22	1471	59	274	26	148	9	437	25	1
Microsomes: Rat Liver												
Vehicle Control		11	1	10	4	14	5	24	2	153	19	1
Test Article	5.00 µg	16	4	6	1	21	4	34	2	145	7	1
	10.0 µg	12	4	9	2	16	4	28	5	139	11	1
	50.0 µg	9	2	10	3	13	2	34	0	155	4	1
	100 µg	15	4	7	3	21	4	39	4	151	17	1
	500 µg	10	3	9	2	17	5	20	7	110	13	2
	1000 µg	15	3	8	3	13	2	15	2	108	13	3
	5000 µg	5	5	0	0	0	0	0	0	45	39	4
Positive Control***		90	11	98	11	1033	117	873	127	800	95	1

** TA1535 sodium azide @ 10 µg/plate
TA1537 quinacrine mustard @ 5 µg/plate
TA1538 2-nitrofluorene @ 10 µg/plate
TA98 2-nitrofluorene @ 10 µg/plate
TA100 sodium azide @ 10 µg/plate

*** TA1535 2-aminoanthracene @ 2.5 µg/plate
TA1537 2-aminoanthracene @ 2.5 µg/plate
TA1538 2-aminoanthracene @ 2.5 µg/plate
TA98 2-aminoanthracene @ 2.5 µg/plate
TA100 2-aminoanthracene @ 2.5 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
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4 = extremely reduced
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mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

[a] The density of the cultures of tester strains TA1537 (0.3×10^9) and TA98 (0.3×10^9) were below the acceptable range ($\geq 0.5 \times 10^9$) However, since all other tester strain characterization indicators were acceptable (culture turbidity, ampicillin and crystal violet sensitivities, and positive and vehicle control values), the data generated with these cultures of TA1537 and TA98 have been accepted

SECTION 3

MUTAGENICITY TESTS ON 1,3-DICHLOROTETRAFLUOROBENZENE IN THE CHO/HGPRT FORWARD MUTATION ASSAY

Young, R.R.[a]

ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of 1,3-dichlorotetrafluorobenzene (DCFB) to induce forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary cells under conditions with and without metabolic activation.

The test material was emulsified in the biologically compatible surfactant Pluronic® F-68 to permit preparation of homogeneous treatment media. DCFB etched polystyrene tissue culture flasks normally used for cell cultivation and treatment. For this reason, cell treatment for the cytotoxicity and mutation assays was performed in sterile glass tissue culture bottles. The cells were exposed to the test material for four hours in the presence and absence of rat liver S9 metabolic activation. The test material remained in an emulsion during the four hour treatment period. Dose-related toxicity was observed that resulted in total cell killing at 1.0 mg/mL. Mutant frequencies of cultures treated with test material varied randomly with dose within ranges comparable to the mutant frequencies of the concurrent vehicle controls. The test material was therefore evaluated as negative for inducing forward mutations at the HGPRT locus in Chinese hamster ovary cells under conditions with and without metabolic activation.

INTRODUCTION

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is a cellular enzyme that allows cells to salvage hypoxanthine and guanine for use in DNA synthesis. The HGPRT enzyme utilizes the substrates 5-phosphoribosyl-1-pyrophosphate and hypoxanthine or guanine to catalyze the formation of inosine- or guanosine monophosphate. If a purine analog such as 6-thioguanine (TG) is included in the growth medium, the analog will be phosphorylated via the HGPRT pathway and incorporated into nucleic acids, eventually resulting in cellular death. The HGPRT locus is located on the X chromosome. Since only one of the two X chromosomes is functional in Chinese hamster ovary (CHO) cells, a single-step forward mutation from HGPRT + to HGPRT- in the functional X chromosome will render the cell unable to utilize hypoxanthine, guanine, or TG supplied

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Such mutants are as viable as wild-type cells in normal medium because DNA synthesis may still proceed by *de novo* synthetic pathways that do not involve hypoxanthine or guanine as intermediates. The basis for the selection of HGPRT- mutants is the loss of their ability to utilize toxic purine analogs (e.g., TG), which enables only the HGPRT- mutants to grow in the presence of TG. Cells which grow to form colonies in the presence of TG are assumed to have mutated, either spontaneously or by the action of the test article, to the HGPRT- genotype.

The objective of this *in vitro* study was to evaluate the ability of 1,3-dichlorotetrafluorobenzene (DCFB) to induce forward mutations at the HGPRT locus in the CHO-K1-BH_4 Chinese hamster ovary (CHO) cell line as assessed by colony growth in the presence of 6-thioguanine (TG). Testing was performed both in the presence and absence of S9 metabolic activation.

MATERIALS AND METHODS

Test Article: 1,3-Dichlorotetrafluorobenzene (DCFB), Lot Number: 01609LV, Physical Description: Clear, colorless liquid

Indicator Cells

The indicator cells used for this study were CHO cells. The hypodiploid CHO-K1 cell line was originally derived from the ovary of a female Chinese hamster (*Cricetulus griseus*) (Kao and Puck, 1968). Characteristics of the cell line were high clonability (approximately 85%) and rapid doubling time (11-14 h). The particular clone used in this assay was CHO-K1-BH_4. The BH_4 subclone of CHO-K1 cells, isolated by Dr. A.W. Hsie (Oak Ridge National Laboratory, Oak Ridge, TN), has been demonstrated to be sensitive to many chemical mutagens.

The CHO-K1-BH_4 cells used in this study were obtained in October 1982 from Dr. Hsie. Master stocks of the cells were maintained frozen in liquid nitrogen. Laboratory cultures were maintained as monolayers at 37 ± 1°C in a humidified atmosphere containing 5% ± 1.5% CO_2. Laboratory cultures were periodically checked for karyotype stability and for the absence of mycoplasma contamination. To reduce the negative control frequency (spontaneous frequency) of HGPRT- mutants to as low a level as possible, the cell cultures were exposed to conditions which selected against the HGPRT- phenotype. Cells were maintained in cleansing medium for two to three days, placed in recovery medium for one day and then returned to culture medium. Cleansed cultures were used to initiate mutation assays from three to seven days after having been removed from cleansing medium.

Media

The cells used during experimental studies were maintained in Ham's Nutrient Mixture F12 supplemented with L-glutamine, antibiotics, and fetal bovine serum (8% by volume), hereafter referred to as culture medium. Cleansing medium used for reducing the spontaneous frequency of HGPRT- mutants prior to experimental studies consisted of culture medium (5% serum) supplemented with 5.0×10^{-6} M thymidine, 1.0×10^{-5} M hypoxanthine, 1.0×10^{-4} M glycine, and 3.2×10^{-6} M of either aminopterin or methotrexate. Recovery medium was similar to cleansing medium except that the aminopterin or methotrexate component was removed and the fetal bovine serum was increased to 8% by volume. Selection medium for mutants was hypoxanthine-free F12 medium containing 4 µg/mL (24 mM) of TG and the fetal bovine serum component reduced to 5% by volume.

Control Articles

Negative (media) controls were performed for each portion of the assay by carrying cells unexposed to the test article through all of the assay operations. In the activation portion of the assay, the negative control cultures were exposed to the S9 metabolic activation mix. A single culture was used in the cytotoxicity assays. A single negative control was added to the activation mutation assay but not in the nonactivation mutation assay.

The test material was a liquid with poor solubility directly in water or culture medium. In order to effectively prepare primary 10X stocks, the test material was emulsified in sterile deionized water that contained 10% (w/v) Pluronic® F-68 (BASF-Wyandotte, lot number WPHB 500B and WPDJ 546B). The primary test material stocks were then diluted 1:10 into culture medium resulting in varying test material concentrations emulsified in 1% Pluronic® F-68 in the treatment medium. Therefore, concurrent vehicle controls were performed for each portion of the study by exposing cells to 1% Pluronic® F-68 in culture medium. In the activation portions of the study, the vehicle controls were also exposed to the rat liver S9 metabolic activation mix. A single culture was used in the cytotoxicity assays and duplicate cultures were used in the mutation assays.

5-Bromo-2'-deoxyuridine (BrdU) is a chemical that is reproducibly and highly mutagenic to CHO-K1 cells without S9 metabolic activation. BrdU (Sigma Chemical Co., lot number 81F-0082) was used at a concentration of 50 µg/mL as a concurrent positive control article for nonactivation mutation studies.

3-Methylcholanthrene (MCA) requires metabolic activation by microsomal enzymes to become mutagenic to CHO-K1-BH_4 cells. MCA (Sigma Chemical Co., lot number 70F-0300) was used at 5 µg/mL as a concurrent positive control article for mutation assays performed with S9 activation.

S9 Metabolic Activation System

The *in vitro* metabolic activation system was comprised of rat liver enzymes (S9 fraction) and an energy producing system, CORE (nicotin-amide adenine dinucleotide phosphate, glucose-6-phosphate and an ion mix) prepared in a phosphate buffer. The enzymes were contained in a 9000 × g supernatant from liver homogenate prepared from Sprague Dawley rats treated with 500 mg/kg of Aroclor 1254 (Molecular Toxicology, Inc., lot number 0249) five days prior to sacrifice. The treatment with Aroclor 1254 was used to induce mixed function oxidase enzymes capable of transforming chemicals to more active forms. The S9 and reaction mixture (CORE) were retained frozen at about -80°C until used. The S9 fraction and CORE were thawed immediately before use and combined to form the activation system described below

Component	Final Concentration in Cultures
NADP (sodium salt)	1.0 mM
Glucose-6-phosphate	5.0 mM
Calcium chloride	2.0 mM
Potassium chloride	6.6 mM
Magnesium chloride	2.0 mM
Phosphate	2.0 mM
S9 homogenate	20.0 µL/mL

The amount of S9 homogenate per culture depends upon the lot of S9 in use at any time. Before use in the assay, each lot of S9 homogenate was tested when purchased. Because the enzymatic activity of S9 homogenate varies among lots, S9 at various concentrations was tested against reference chemicals such as benzo(a)pyrene or 3-methylcholanthrene. The optimum S9 concentration was selected based on induction of HGPRT- mutants in CHO cells, and this amount of S9 was used in all subsequent assays with that particular lot of S9.

Dosing Procedure

In order to achieve as uniform an exposure as possible for cell monolayers treated with the test material in culture medium, Pluronic® F-68 was investigated as an emulsifying agent for the test material. Pluronic® F-68 is a polyalcohol that is frequently used in cell cultures due to its low toxicity and ability to lower surface tension. When a preparation of 50 mg/mL of DCFB in 10% w/v Pluronic® F-68 in deionized water was vigorously agitated for about one minute with a Tissumizer® at a setting of 40, a stable, white emulsion was formed. The DCFB remained dispersed into tiny droplets after diluting 1:10 into culture medium and did not coalesce upon sitting.

A fresh emulsion of DCFB was prepared in 10% w/v Pluronic® F-68 for each dose rangefinding study and mutation assay. A Tissumizer® was used at a setting of 40-50 for 45 sec. to 1 min. to prepare the emulsions. The initial concentration of DCFB was prepared at either 10 or 50 mg/mL, and lower

concentrations were prepared by diluting the emulsions into 10% w/v Pluronic® F-68. The emulsions were mixed well by vortex before preparing the treatment media. The treatment media were prepared as 1:10 dilutions of the emulsions into F12 culture medium so that the Pluronic® F-68 content was diluted to 1% w/v.

Rangefinding Cytotoxicity Testing

After the selection of 10% w/v Pluronic® F-68 as a suitable vehicle, a wide range of test article concentrations was tested for cytotoxicity both with and without S9 metabolic activation. Ten concentrations that spanned a three-log concentration range were used. The applied doses ranged from 0.005 to 5.0 mg/mL. In addition, one negative (media) control and one vehicle control containing 1% w/v Pluronic® F-68 were used in each cytotoxicity assay.

Glass culture bottles with approximately 60 cm^2 of surface area were seeded at 200 cells per flask, allowed to attach overnight (16 to 18 h) and exposed to the test or control article for four hours. A single culture was used for each test or control treatment condition. The cells were then washed twice with Dulbecco's phosphate buffered saline (PBS) and incubated in F12 culture medium for six additional days to allow colony development. Colonies were then fixed in alcohol, stained with Giemsa and counted by eye, excluding those with approximately 50 cells or less. Cytotoxicity was expressed as a percentage of colony counts in treated cultures versus control culture. The preliminary cytotoxicity information was used to select doses for the mutation assays that covered a range of toxicity from nontoxic to highly toxic.

Nonactivation Mutagenicity Testing

The assay procedure was based on that reported by Hsie et al. (1975), and reviewed by Hsie et al. (1981), with modifications suggested by Myhr and DiPaolo (1978). The cleansed cells were plated at about 2.4×10^6 cells per 60 cm^2 glass tissue culture bottle on the day before dosing. The time between plating and treatment was about 18 h. Cell cultures were treated with test or control material for four hours. Cell cultures normally contain at least 4×10^6 cells by the time of treatment termination. After treatment, the cell monolayers were washed twice with phosphate buffered saline, trypsinized, and suspended in culture medium. The cell suspension from each dose was counted using a Coulter Counter and replated at 1.5×10^6 cells into each of two 150-mm dishes and at 200 cells into each of three 60-mm dishes. The small dishes were incubated for seven days to permit colony development and the determination of the cytotoxicity associated with each treatment. The large dishes were incubated for seven days to permit growth and expression of induced mutations. The mass cultures were subcultured every two or three days during the expression period to maintain logarithmic cell growth. At each subculture the cells from the two 150-mm dishes from each dose were combined and reseeded at about 1.5×10^6 cells into each of two 150-mm dishes.

At the end of the expression period (seven days), each culture was reseeded at 2×10^5 cells per 100-mm dish (12 dishes total) in mutant selection medium. Also, three 60-mm dishes were seeded at 200 cells each in culture medium to determine the cloning efficiency of each culture. After incubation for seven to ten days, at 37 ± 1°C in a humidified atmosphere with about 5% CO_2, the colonies were fixed with alcohol, stained with Giemsa and counted to determine the number of TG-resistant colonies in mutant selection dishes and the number of colonies in the cloning efficiency dishes. The colonies were counted by eye, excluding those with approximately 50 cells or less.

Activation Mutagenicity Assay

The activation assay was performed independently with its own set of vehicle and positive controls. The procedure was identical to the nonactivation assay except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors during the four-hour treatment period. The fetal bovine serum content of the medium used for dosing was reduced to 5% by volume. The cofactors consisted of nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), glucose-6-phosphate, calcium chloride, potassium chloride, and magnesium chloride, all of which were in a pH 7.8 sodium phosphate buffer.

Data Presentation

The collected data were used to calculate several assay parameters. The chosen combination of raw data and calculated data allows a complete description of events for each treatment condition. The significance of each calculated parameter and its method of calculation are listed below

Relative Survival to Treatment: This parameter gives the clonal cytotoxicity of each treatment by showing what percentage of the cells were able to form colonies after the treatment period in both the rangefinding cytotoxicity assays and the mutation assays relative to the concurrent vehicle controls. The average number of colonies in three dishes (seeded at 200 cells each) was determined for each treatment condition.

$$\text{Relative Survival (\%)} = \frac{\text{Average no. of colonies per treated culture}}{\text{Average no. of colonies per vehicle control dish}} \times 100\%$$

Relative Population Growth: This parameter shows the cumulative growth of the treated cell population, relative to the vehicle control growth, over the entire expression period and prior to mutant selection. In general, highly toxic treatments will reduce the growth rate as well as the survival

Values less than 100% indicate growth inhibition. For example, 50% and 25% relative growth values would indicate treated cell populations that were one and two population doublings behind

the negative control culture. Treated populations that are more than two or three doublings behind the control might not achieve maximum expression of the TG-resistant phenotype. The relative population growth is calculated from cell count data not presented in this report and is intended to provide only an approximate indication of growth during the expression period, since cells are easily lost or not completely released by trypsin during the subculture procedures.

$$\text{Relative Population Growth (\%)} = \frac{\text{Treated culture population increase over the expression period}}{\text{Vehicle control population increase over the expression period}} \times 100\%$$

Absolute Cloning Efficiency: The ability of the cells to form colonies at the time of mutant selection is measured by the absolute cloning efficiency (CE). This parameter is used as the best estimate of the cloning efficiency of the mutant cells in the selection dishes. Thus, the observed number of mutant colonies can be converted to the frequency of mutant cells in the treated population.

$$\text{Absolute CE (\%)} = \frac{\text{Average no. of viable colonies per dish}}{200} \times 100\%$$

Mutant Frequency: The mutant frequency is the endpoint of the assay. It is calculated as the ratio of colonies found in thioguanine-selection medium to the total number of cells seeded, adjusted by the absolute CE. The frequency is expressed in units of 10^{-6}, eg., the number of mutants per one million cells.

$$\text{Mutant Frequency} = \frac{\text{Total mutant clones}}{\text{no. of dishes} \times 2 \times 10^5 \times \text{abs. CE}}$$

Assay Acceptance Criteria

An assay normally is considered acceptable for evaluation of the results only if all of the following criteria are satisfied. The activation and nonactivation portions of the mutation assay may be performed concurrently, but each portion is, in fact, an independent assay with its own positive and vehicle controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

- The average absolute cloning efficiency of the vehicle controls should be between 70% and 115%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and dilutions during cloning. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50 to 70% and still yield internally consistent and valid

results. Assays with cloning efficiencies in this range will be conditionally acceptable and dependent on the scientific judgment of the Study Director. All assays below 50% cloning efficiency will be unacceptable

- The background mutant frequency (average of the vehicle controls) is calculated separately for the activation and non-activation assays, even though the same population of cells may be used for concurrent assays. The activation vehicle controls contain the S9 activation mix and may have a slightly different mutant frequency than the nonactivation vehicle controls. For both conditions, background frequencies for assays performed with different cell stocks are generally 0 to 10×10^{-6}. Assays with backgrounds greater than 15×10^{-6} will not be used for evaluation of a test article.

- A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria. If the test article appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency that is significantly elevated over the concurrent vehicle controls ($p \leq 0.01$).

- For test articles with little or no mutagenic activity, an acceptable assay should include applied concentrations that reduce the clonal survival to approximately 10% to 15% of the average of the vehicle controls, reach the maximum applied concentrations given in the evaluation criteria, reach a concentration that is approximately twice the solubility limit of the test article in culture medium or include a high concentration that is at least 75% of an excessively toxic concentration. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.

- Mutant frequencies are normally derived from sets of 12 dishes for the mutant colony count and three dishes for the viable colony count. To allow for contamination losses, an acceptable mutant frequency for treated cultures can be calculated from a minimum of eight mutant selection dishes and two cloning efficiency dishes.

- The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions in order to accept a single assay for evaluation of the test article.

Assay Evaluation Criteria

Mutation assays are initiated by exposing cell cultures to about six to eight concentrations of test article that are expected, on the basis of preliminary toxicity studies, to span a range of cellular responses from no observed toxicity to about 10% survival. Five doses are usually then selected for completion of the mutation assay. These doses should cover a range of toxicities with emphasis placed on the most toxic doses. An assay may need to be repeated with different concentrations to properly evaluate a test article.

The statistical tables provided by Kastenbaum and Bowman (1970) are used to determine whether the results at each dose are significantly different from the negative controls at 95% or 99% confidence levels. This test compares variables distributed according to Poissonian expectations by summing up the probabilities in the tails of two binomial distributions. The 95% confidence level must be met as one criterion for considering the test article to be active at a particular dose. In addition, the mutant frequency must meet or exceed 15×10^{-6} in order to compensate for random fluctuations in the 0 to 10×10^{-6} background mutant frequencies that are typical for this assay.

Observation of a mutant frequency that meets the minimum criteria for a positive response in a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test article as a mutagen. The following test results must be obtained to reach this conclusion for either activation or non-activation conditions:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses. However, this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears. If an increase in mutant frequency is observed for a single dose near the highest testable toxicity, as defined previously, and the number of mutant colonies is more than twice the value needed to indicate a significant response, the test article generally will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

- For some test articles, the correlation between toxicity and applied concentration is poor. The proportion of the applied article that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent survival), can be used to establish whether the mutagenic activity is related to an increase in effective treatment.

A test article is evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to concentrations causing about 10% to 15% survival or extends to a concentration at least 75% of that causing excessive toxicity. If the test article is relatively nontoxic, the maximum applied concentrations will normally be 5 mg/mL (or 5 µL/mL) for water-soluble materials or 1 mg/mL (or 1 µL/mL) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response as discussed above, the test article is evaluated as nonmutagenic in this assay system.

This presentation may not encompass all test situations, and the Study Director may use other criteria to arrive at a conclusion, especially when data from several repeat assays are available. The interpretation of the results in the Results and Discussion section provides the reasoning involved when departures from the above descriptions occur.

RESULTS AND DISCUSSION

Test Material Handling

The test material, DCFB, was found to be soluble in dimethylsulfoxide (DMSO) at 100 mg/mL. The 100 mg/mL stock solution was diluted 1:10 and 1:100 into culture medium. Immiscible globules of test material formed at 10 and 1 mg/mL. DMSO was not a suitable solvent for preparing treatment media. In order to achieve as uniform an exposure as possible for cell monolayers treated with the test material in culture medium, Pluronic® F-68 was investigated as an emulsifying agent for the test material. The surfactant Pluronic® F-68, manufactured by BASF Wyandotte, was used to prepare emulsions of the sample for use as 10X primary stocks. The Pluronic® F-68 was prepared at ten percent w/v in sterile deionized water followed by filtration through a 0.45 µM filter. The initial stock for each assay was prepared by adding together the desired weight of test material and volume of Pluronic® F-68. Stable emulsions were produced after this preparation was homogenized using a Tissumizer®. Lower 10 x stocks were prepared by dilution with 10% Pluronic® F-68 solution.

Rangefinding Cytotoxicity Assay

The sample, DCFB, was tested in the preliminary rangefinding cytotoxicity assay with and without S9 metabolic activation Ten doses were used in each case that ranged from 0.005 to 5.0 mg/mL.

The rangefinding cytotoxicity assay showed that the test material was toxic to CHO cells in culture both with and without S9 metabolic activation (Tables 3-1 and 3-2). Without activation, the test material was nontoxic from 0.005 to 0.02 mg/mL, followed by increased toxicity at 0.05 mg/mL. Total cell killing was obtained at 0.1 mg/mL and higher. With activation, no toxicity was observed from 0.005 to 0.05 mg/mL. Total, or near-total, cell killing was obtained at 0.1 mg/mL and higher.

The results from the preliminary rangefinding cytotoxicity assays were used to select ten doses for the mutation assays. Treatment conditions chosen for the nonactivation portion of the mutation assay covered a 200-fold range from 0.005 to 1.0 mg/mL. With activation, doses selected covered a 100-fold range from 0.01 to 1.0 mg/mL.

Mutation Assay Without Metabolic Activation

Under nonactivation test conditions, the cultures treated with DCFB showed a dose-related decrease in both relative survival and relative population growth (Table 3-3). The culture exposed to the highest concentration, 1.0 mg/mL, was completely toxic and was terminated. Of the remaining nine doses, the lowest dose, 0.005 mg/mL, was not plated for mutant selection due to a sufficient number of surviving nontoxic doses. Eight doses were available for analysis.

Without S9 metabolic activation, the mutant frequency of cultures treated with the test material varied within the acceptable range of vehicle control mutant frequency variation which is 0 to 15 $\times$ 10^{-6}. There was no positive correlation of mutant frequency with dose and no treated culture had a mutant frequency that was significantly elevated over the average background mutant frequency of the concurrent vehicle controls. Therefore, DCFB was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells in the absence of S9 metabolic activation.

The positive control treatment with 50 µg/mL BrdU induced a large, significant ($p \leq 0.01$) increase in mutant frequency. The background mutant frequencies of the two vehicle controls were acceptable. The assay results achieved all assay acceptance criteria, which provided confidence in the assumption that the recorded data represented a typical response of the test material in the nonactivation assay system.

Mutation Assay With Metabolic Activation

Under S9 metabolic activation test conditions, the cultures treated with DCFB showed a dose-related decrease in both relative survival and relative population growth (Table 3-4). The cultures exposed to the two highest concentrations, 0.5 and 1.0 mg/mL, were excessively toxic and were terminated prior to plating for mutant selection. Of the remaining eight doses, the next to the lowest dose, 0.025 mg/mL, was not plated for mutant selection due to a sufficient number of surviving nontoxic doses. Seven doses were available for analysis.

With S9 metabolic activation, the mutant frequency of cultures treated with the test material varied within the acceptable range of vehicle control mutant frequency variation which is 0 to 15 $\times$ 10^{-6}. There was no positive correlation of mutant frequency with dose and no treated culture had a mutant frequency that was significantly elevated over the average background mutant

frequency of the concurrent vehicle controls. Therefore, DCFB was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells in the presence of S9 metabolic activation.

The positive control treatment with 5 µg/mL MCA induced a large, significant ($p \leq 0.01$) increase in mutant frequency which demonstrated the effectiveness of the S9 metabolic activation system and the ability of the test system to detect known mutagens. The background mutant frequencies of the negative control and the 1% Pluronic® F-68 vehicle controls were within the acceptable range and comparable to the historical activation control data (see Appendix 3-A). The assay results achieved all assay acceptance criteria and provided confidence in the assumption that the recorded data represented typical responses of the test material in the assay system.

CONCLUSION

The test material, DCFB, is considered negative for inducing forward mutations at the HGPRT locus in CHO cells under both the S9 metabolic activation and non-activation conditions of the assay.

REFERENCES

Hsie, A.W., P.A. Brimer, T.J. Mitchell, and D.G. Gosslee. 1975. Dose-response relationship for ethyl methanesulfonate-induced mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells. *Somat. Cell Genet.*, 1(3):247-261.

Hsie, A.W., D.A. Casciano, D.B. Couch, D.F. Krahn, J.P. O'Neil, and B.L. Whitfield. 1981. The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. *Mutation Res.*, 86:193-214.

Kao, F.T. and T.T. Puck. 1968. Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. *Proc. Natl. Acad. Sci. U.S.*, 60(4):1275-1281.

Kastenbaum, M.A. and K.O. Bowman. 1970. Tables for determining the statistical significance of mutation frequencies. *Mutation Res.*, 9:527-549.

Myhr, B.C. and J.A. DiPaolo. 1978. Mutagenesis of Chinese hamster cells *in vitro* by combination treatments with methyl methanesulfonate and N-acetoxy-2-acetylaminofluorene. *Cancer Res.*, 38:2539-2543.

TABLE 3-1. CLONAL CYTOTOXICITY ASSAY OF DCFB WITHOUT METABOLIC ACTIVATION

APPLIED CONCENTRATION (MG/ML)	NUMBER OF COLONIES	RELATIVE SURVIVAL[a] (PERCENT)	CLONING EFFICIENCY (PERCENT)
NC[b]	159	88.3	
VC, 1%[c]	180	100.0	90.0
DCFB:			
0.005	164	91.1	
0.01	202	112.2	
0.02	173	96.1	
0.05	45	25.0	
0.1	0	0.0	
0.2	0	0.0	
0.5	0	0.0	
1.0	0	0.0	
2.0	0	0.0	
5.0	0	0.0	

[a]Relative to vehicle control
[b]NC = Negative control, F12 medium
[c]VC = Vehicle control, 1% Pluronic® F-68

TABLE 3-2. CLONAL CYTOTOXICITY ASSAY OF DCFB WITH S9 METABOLIC ACTIVATION

APPLIED CONCENTRATION (MG/ML)	NUMBER OF COLONIES	RELATIVE SURVIVAL[a] (PERCENT)	CLONING a EFFICIENCY (PERCENT)
NC[b]	178	100.6	
VC, 1%[c]	177	100.0	88.5
DCFB:			
0.005	167	94.4	
0.01	181	102.3	
0.02	170	96.0	
0.05	152	85.9	
0.1	4	2.3	
0.2	0	0.0	
0.5	0	0.0	
1.0	0	0.0	
2.0	0	0.0	
5.0	0	0.0	

[a] Relative to vehicle control
[b] NC = Negative control, F12 medium
[c] VC = Vehicle control, 1% Pluronic® F-68

TABLE 3-3. MUTATION ASSAY OF DCFB WITHOUT METABOLIC ACTIVATION - TRIAL I

NONACTIVATION TEST CONDITION	SURVIVAL TO TREATMENT (% VEH. CONTROL)	RELATIVE POPULATION GROWTH (% OF CONTROL)	TOTAL MUTANT COLONIES	ABSOLUTE C.E. ± S.D. (%)	MUTANT FREQ IN 10^{-6} UNITS[a]
Vehicle Control[b]	95.9	92.2	15	103.0 ± 4.6	6.1
Vehicle Control	104.1	107.8	15	84.0 ± 1.8	7.4
Positive Control (50 µg/mL BrdU)[c]	71.6	43.0	228	90.2 ± 2.1	105.3[d]
DCFB (mg/mL)					
0.005	89.7	NS[e]	—	—	—
0.01	98.2	105.5	10	86.2 ± 2.4	4.8
0.02	98.0	99.5	2	88.0 ± 11.3	0.9
0.03	92.2	110.8	11	93.0 ± 1.5	4.9
0.04	95.7	104.8	7	100.5 ± 2.0	2.9
0.05	92.0	94.5	7	98.4 ± 4.5	3.0
0.06	77.8	111.4	16	98.9 ± 3.0	6.7
0.08	41.8	79.7	16	101.5 ± 7.8	6.6
0.1	22.7	57.0	9	103.4 ± 6.2	3.6
1.0	0.0	T[f]	—	—	—

[a]Mutant Frequency = Total mutant colonies/ (No. of dishes × 2 × 10^5 × absolute C.E.)

[b]VC = Vehicle Control. 1% Pluronic® F-68

[c]BrdU = 5-Bromo-2'-deoxyuridine

[d]Significant increase. Kastenbaum Bowman test $p \leq 0.01$ and mutant frequency $\geq 15 \times 10^{-6}$

[e]NS = Not plated for selection due to sufficient surviving higher dose levels

[f]T = Terminated due to excessive toxicity

TABLE 3-4. MUTATION ASSAY OF DCFB WITH METABOLIC ACTIVATION - TRIAL I

ACTIVATION TEST CONDITION	SURVIVAL TO TREATMENT (% VEH. CONTROL)	RELATIVE POPULATION GROWTH (% OF CONTROL)	TOTAL MUTANT COLONIES	ABSOLUTE C.E. ± S.D. (%)	MUTANT FREQ IN 10^{-6} UNITS[a]
Negative Control[b]	98.0	109.3	7	100.4 ± 4.6	2.9
Vehicle Control[c]	104.7	103.6	11	97.0 ± 4.1	4.7
Vehicle Control	95.3	96.4	5	103.0 ± 8.2	2.0
Positive Control (5 µg/mL 3-MCA)[d]	82.3	60.8	715	105.0 ± 6.9	283.7[e]
DCFB (mg/mL)					
0.01	104.2	90.9	9	105.2 ± 7.6	3.6
0.025	98.0	NS[f]	—	—	—
0.05	106.6	101.8	9	103.4 ± 2.5	3.6
0.07	97.1	61.0	16	105.5 ± 3.1	6.3
0.08	94.6	84.4	12	108.3 ± 5.8	4.6
0.09	89.2	81.3	20	98.2 ± 2.8	8.5
0.1	40.2	42.9	5	86.5 ± 7.4	2.4
0.25	2.8	5.2	3	94.0 ± 7.3	1.3
0.50	0.5	T[g]	—	—	—
1.0	0	T[g]	—	—	—

[a]Mutant Frequency = Total mutant colonies (No. of dishes × 2 × 10^5 × absolute C.E.)

[b]NC = Negative (Media) Control

[c]VC = Vehicle Control 1% Pluronic® F-68

[d]3-MCA = 3-Methylcholanthrene

[e]Significant increase Kastenbaum Bowman test $p \leq 0.01$ and mutant frequency $\geq 15 \times 10^{-6}$

[f]NS = Not selected due to sufficient nontoxic dose levels

[g]T = Terminated due to excessive toxicity

APPENDIX 3-A

HISTORICAL CHO HGPRT ASSAY CONTROL MUTANT FREQUENCY DATA

A. Nonactivation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$3.9 \pm 2.9 \times 10^{-6}$
Range	0 to 16.8×10^{-6}
Number of experiments	50
Number of controls	88

2. Positive controls (50 µg/mL 5-bromo-2'-deoxyuridine)

Mean (± SD)	$121.6 \pm 27.9 \times 10^{-6}$
Range	38.7 to 165.6×10^{-6}
Number of experiments	50
Number of controls	59

B. Activation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$2.9 \pm 2.1 \times 10^{-6}$
Range	0 to 10.0×10^{-6}
Number of experiments	50
Number of controls	86

2. Positive controls (5 µg/mL 3-methylcholanthrene)

Mean (± SD)	$370.0 \pm 173.3 \times 10^{-6}$
Range	152.3 to 941.6×10^{-6}
Number of experiments	50
Number of controls	61

The historical control data was compiled from the most recent fifty experiments. Because some experiments contained duplicate controls, the number of independent control cultures exceeded the number of experiments.

SECTION 4

IN VITRO CYTOGENETIC ASSAY MEASURING SISTER CHROMATID EXCHANGE AND CHROMOSOMAL ABERRATION FREQUENCIES IN CHINESE HAMSTER OVARY CELLS TREATED WITH 1,3-DICHLOROTETRAFLUOROBENZENE

Murli, H.[a]

ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of 1,3-dichlorotetrafluorobenzene (DCFB) to induce sister chromatid exchange (SCE) and chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation. In the SCE assay, single cultures of CHO cells were incubated with 0.167 to 5010 µg/mL in a half-log series. Complete cellular toxicity was observed at 167, 501, 1670, and 5010 µg/mL in both activation and nonactivation trials. A weak increase in SCE was observed at 50.1 µg/mL, the highest analyzable dose in both assays. This response was not repeated in a subsequent trial that tested concentrations of 10.0 to 100 µg/mL under activation and nonactivation conditions

Based on the evaluation of cell cycle kinetics from the SCE assay, a 10 h harvest was selected for testing concentrations of 6.26 to 25.1 µg/mL and a 20 h harvest was selected for testing concentrations of 25.0 to 100 µg/mL in the nonactivation aberrations assay, and a 10 h harvest was selected for testing doses of 7.51 to 150 µg/mL in the aberrations assay with activation. The nonactivation aberrations assay was repeated testing the same concentrations due to ambiguous toxicity in the first trial. Replicate cultures of CHO cells were dosed in the aberrations assays. No increase in chromosomally aberrant cells was observed at the concentrations analyzed.

The test article, DCFB, is considered negative for inducing SCE and for inducing chromosomal aberrations in CHO cells under both nonactivation and activation conditions of this assay.

INTRODUCTION

Sister chromatid exchanges (SCE) are seen at metaphase as reciprocal interchanges of the two chromatid arms within a single chromosome. These exchanges presumably require enzymatic incision, translocation, and ligation of the two DNA strands. The frequency of SCE is thought to be a very sensitive indicator of damage to the genetic material, DNA. Increases in the frequency of SCE are

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caused by many chemical agents known to be mutagens/carcinogens. Thus SCE test is a relevant cytogenetic test for potentially genotoxic chemicals.

The SCE test involves treating cultured cells with a test compound, growing cells with the thymidine analog 5-bromo-2'-deoxyuridine (BrdUrd) for ~2 cell cycles, and making chromosome preparations that are stained for SCE. The chromosomes of dividing cells consist of two identical halves, or sister chromatids. By growing cells with BrdUrd for two cell cycles one chromatid contains half as much BrdUrd as the other and is stained more intensely by Giemsa, while its pair, or sister, is pale.

This assay is also designed to establish whether the test article or its metabolites can interact with cells to induce chromosomal breaks. Chemically induced lesions may result in breaks in chromatin that are either repaired by the cell in such a way as to be undetectable or result in visible damage. Aberrations are a sequence of failure or mistakes in repair processes such that breaks do not rejoin or rejoin in abnormal configurations (Evans, 1962).

The chromosomal aberrations assay is designed to examine cells in the first mitosis after chemical exposure. This design limits loss of aberrant cells during the division process or conversion into complex derivatives during subsequent cell cycles. In the case of Chinese hamster ovary (CHO) cells most dividing cells examined 8 to 12 h after treatment are in the first mitosis (M1 cells). However, many test articles cause severe delay of progression through the cell cycle, and the assay has been designed to detect this delay and allow for slower growth of damaged cells by adjustments in the time between treatment and cell fixation.

The objective of this *in vitro* assay was to evaluate the ability of 1,3-dichlorotetrafluorobenzene (DCFB) to induce SCE and chromosomal aberrations in CHO cells, with and without metabolic activation.

EXPERIMENTAL DESIGN

In the SCE assay, CHO cell cultures which were exposed to the test article for approximately two cell cycles were analyzed to determine cellular toxicity and effects of the test article on cell generation time. If necessary and possible, the assay was extended in cultures at affected doses to allow for the progression to second generation cells. The doses used in the assay ranged from 0.167 µg/mL of the test article solution through 5010 µg/mL in a half-log series. Single cultures were used for the negative control, solvent control, each of two doses of the positive control and 10 doses of the test material. A second trial was conducted to verify the response from the first trial, and concentrations of 10.0 to 100 µg/mL were tested in this trial. Sister chromatid exchange frequencies were analyzed from cultures treated with the four highest doses with second generation cells and

from a negative, solvent and positive control culture. Cell cycle kinetics of the treated cultures were also evaluated.

Summary of SCE Assay Treatment Schedule in Hours

Test	Test Article	Wash	BrdUrd	Wash	Colcemid®	Fixation
-S9	-2.25		0	22.75	23	25.5
+S9	-2.25	-0.25	0		23	25.5

Cell cycle kinetics from the SCE assay were used 1) to determine the dose range to be used in the chromosomal aberrations assay and 2) to determine the optimal time of harvest of the dosed cells so that primarily metaphase cells which were in the first metaphase since exposure to the test article would be analyzed for chromosomal aberrations. The aberrations assay was conducted at the 10 h harvest time for those chemicals which did not induce any cell cycle delay and at the 20 h harvest time for those chemicals that induced cell cycle delay.

In the chromosomal aberrations assays duplicate cultures were used at each dose. Single cultures were used for the negative control, solvent control, and at each of two doses of the positive control. In the nonactivation assay, 10 and 20 h harvests were conducted. In the activation assay, 10 h harvest was conducted. Chromosomal aberrations were analyzed from the four (nonactivation assay) and five (activation assay) highest doses from which results could be obtained and from only one of the positive control doses. A summary of the treatment schedule for the chromosomal aberrations assays is given below.

Summary of Chromosomal Aberrations Assay Treatment Schedule in Hours

Test	Test Article	Wash	Colcemid®	Fixation
-S9	0	7.25	7.5	10
-S9	0	17.25	17.5	20
+S9	0	2	7.5	10

MATERIALS AND METHODS

Test Material

1,3-Dichlorotetrafluorobenzene, 99%, Lot 01609LV, clear, colorless liquid.

Indicator Cells

The CHO cells (CHO-WBL) used in this assay were from a permanent cell line and were originally obtained from the laboratory of Dr. S. Wolff, University of California, San Fransisco. The cells have since been recloned to maintain karyotypic stability. This cell line has an average cycle time of 12 to 14 h with a modal chromosome number of 21.

Cell Culture Medium

The CHO cells were grown in McCoy's 5a culture medium which was supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin and streptomycin, at about 37°C, in an atmosphere of about 5% CO_2 in air.

Negative and Solvent Controls

In the nonactivation assays, negative controls were cultures which contain only cells and culture medium. Solvent controls were cultures containing the solvent for the test article at the same concentration used in test cultures. In the activation assays, the negative and solvent controls were as in the nonactivation assays but the S9 activation mix was also added.

Positive Control Agents

The positive control agents which were used in the assays were mitomycin C (MMC) for the nonactivation series and cyclophosphamide (CP) in the metabolic activation series. Mitomycin C is a clastogen that does not require metabolic activation. Cyclophosphamide does not act directly but must be converted to active intermediates by microsomal enzymes. In the SCE assay two doses of MMC (0.005 and 0.010 µg/mL) and CP (1.50 and 2.00 µg/mL) were used. In the chromosomal aberrations assays two concentrations of MMC (0.500 and 1.00 µg/mL, 10 h harvest; 0.040 and 0.080 µg/mL, 20 h harvest) and CP (25.0 and 50.0 µg/mL) were used to induce chromosomal aberrations in the CHO cells. Only one dose of the positive control was actually analyzed in each of the SCE and aberration assays.

Sister Chromatid Exchange Assays

In these assays, the cells were cultured for approximately 24 h prior to treatment by seeding approximately 0.8×10^6 cells per 75 cm^2 flask into 10 mL of complete McCoys 5a culture medium. The thymidine analog, BrdUrd, was added at a final concentration of 10 µM approximately two hours after the initial exposure of the cells to the test article.

Nonactivation Assay

The cultures were dosed with the test article for ~two hours when BrdUrd was added at a final concentration of 10 µM. The cultures were then reincubated for approximately 23 h.

Approximately 2.75 h prior to the harvest of the cells, the test article was washed from the cells with phosphate buffered saline and fresh complete medium with BrdUrd (10 µM), and Colcemid® (final concentration 0.1 µg/mL) was added.

Prior to the harvest of the cultures visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures were also evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. Only flasks from the highest six surviving doses from which metaphase cells for analysis were expected were harvested (See Section on Harvest). Medium was collected and the cultures centrifuged. The mitotic cells were fixed but medium was replaced on the remaining cell monolayer in the flasks. A test slide was made from fixed cells treated with the highest doses of test compound, and stained with Hoechst 33258 stain (0.5 µg/mL in phosphate buffer, pH 6.8). The slides were examined under UV fluorescence microscopy. As there was marked cell cycle delay, a second cell collection was made. After ~6.5 h further incubation, the next set of cells to reach mitosis was harvested from the cultures as delayed fixation was required at 16.7 and 50.1 µg/mL. The harvested cells were differentially stained for the analysis of SCE using a modified fluorescence-plus-Giemsa (FPG) technique (See Sections on Harvest and Slide Preparation and Staining).

Assay with Metabolic Activation

In this assay, the CHO cells were exposed to the test article for two hours in the presence of a rat liver S9 reaction mixture (S9 15 µL/mL, NADP 1.5 mg/mL, and isocitric acid 2.7 mg/mL). The S9 fraction was derived from the liver of male Sprague-Dawley rats which had been previously treated with Aroclor 1254 to induce the mixed function oxidase enzymes which are capable of metabolizing chemicals to more active forms. The two hour incubation time was used because prolonged exposure to the S9 mixture might be toxic to the cells and the enzyme activity of S9 is lost rapidly at 37°C. The medium did not have fetal calf serum (FCS) during the exposure period to avoid possible inactivation of short-lived and highly reactive intermediates produced by the S9 enzymes by binding to serum proteins.

In this assay, the CHO cells were incubated at 37°C for two hours in the presence of the test article and the S9 reaction mixture in the growth medium without FCS. After the exposure period the cells were washed twice with buffered saline. Complete McCoys 5a medium with 10 µM BrdUrd was added to the cultures which were then incubated for approximately 23 h. Colcemid® (final concentration 0.1 µg/mL) was then added and the cultures were then reincubated for 2.5 h, harvested and examined for any cell delay. Slides were prepared and

stained as described for the nonactivation assay. Delayed fixation was not required for any of the surviving cultures.

Nonactivation Aberrations Assays

Cultures were initiated by seeding approximately 1.0 x 10^6 cells (20 h assay) and 1.5 x 10^6 cells (10 h assay) per 75 cm^2 flask into 10 mL of complete McCoys 5a medium. One day after culture initiation, the CHO cells to be used in the nonactivation trial were treated with the test article at predetermined doses for 7.25 and 17.25 h. The cultures were then washed with buffered saline and complete McCoys 5a medium containing 0.1 µg/mL Colcemid® was placed back onto the cells. Two and one half hours later the cells were harvested and air dried slides were made. The slides were then stained in pH 6.8 buffered 5% Giemsa solution for the analysis of chromosomal aberrations.

Aberrations Assays with Metabolic Activation

Cultures were initiated by seeding approximately 1.5 x 10^6 cells per 75 cm^2 flask into 10 mL of complete McCoys 5a medium. One day after culture initiation, the cultures that were treated under the conditions of metabolic activation were incubated at 37°C for two hours in the presence of the test article and the S9 reaction mixture in McCoys 5a medium without FCS. After the two hour exposure period the cells were washed twice with buffered saline and the cells were refed with complete McCoys 5a medium. The cells were incubated for an additional 7.75 h with 0.1 µg/mL Colcemid® present during the last 2.5 h of incubation. The metaphase cells were then harvested and prepared for cytogenetic analysis.

Harvest Procedure

Prior to the harvest of the cultures visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures were also evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. The metaphase cells were collected by mitotic shake-off (Terasima and Tolmach, 1961) and were treated with 0.075 M KCl hypotonic solution. This treatment helps to swell the cells and thus disperse the chromosomes. The cultures were then fixed with an absolute methanol:glacial acetic acid (3:1) fixative and were washed several times before air-dried slides were prepared.

Slide Preparation and Staining

Slides were prepared by dropping the harvested cultures on clean slides. The slides from the rangefinding assays were differentially stained using a modified FPG technique (after Perry and Wolff, 1974; Goto et al., 1978). The slides were stained in Hoechst 33258 stain, exposed to ultraviolet light, and then stained with Giemsa Azure B stain. The slides prepared from the aberrations assay

were stained with pH 6.8 buffered 5% Giemsa solution for the analysis of chromosomal aberrations. All slides were then air-dried and coverslipped using Depex® mounting medium.

SCE Analysis and Assay Evaluation

Fifty cells per dose were analyzed from each of the top four doses from which sufficient M2 metaphase cells were available. Fifty cells were read from each of the negative and solvent controls, and at least twenty cells were read from one dose of the positive control. For control of bias, all slides except for the positive controls were coded prior to analysis. Cells were selected for scoring on the basis of good morphology and clear sister chromatid differentiation along the entire length of all chromosomes; only cells with the number of centromeres equal to the modal number 21 ± 2 (range of 19-23) were analyzed.

The slides were examined for the presence of delayed cells. One hundred metaphase cells were scanned and classified as M1, M1+, or M2 from each dose and the positive, negative, and solvent controls to give an estimate of cell cycle inhibition. In those doses where more than one harvest was carried out, cells were analyzed for cell cycle kinetics and SCE from the earliest harvest time from which sufficient M2 cells were available for analysis. Controls were analyzed only at the normal harvest time (25-26 h).

Data were collected on standard forms. The data were summarized in tables showing the numbers of cells scored, total SCEs, SCE per chromosome, and SCE per cell. The cell cycle kinetics were also calculated.

If an increase in SCE was observed, one of the following criteria must normally be met to assess the compound as positive.

Two-fold increase:

Approximately a doubling in SCE frequency over the ***"background" (solvent and negative control) levels at one or more doses.***

Dose response:

A positive assessment may be made in the absence of a doubling if there was a statistically significant increase at a minimum of three doses and evidence for a positive dose response

In some cases, statistically significant increases were observed with neither a doubling nor a dose response. These results were assessed according to repeatability, the magnitude of the response, and the proportion of the doses affected.

Statistical analysis employed a Student t-test (Bancroft, 1957; Hollander and Wolfe, 1973) to compare SCE frequencies in the tested cultures with the negative and solvent controls. The final evaluation of the test article was based upon scientific judgment.

Aberrations Analysis and Assay Evaluation

Cells were selected for good morphology and only cells with the number of centromeres equal to the modal number 21 ± 2 (range 19-23) were analyzed.

One hundred cells, if possible, from each duplicate culture at four (nonactivation assay) and five (activation assay) doses of the test article and from each of the negative and solvent control cultures were analyzed for the different types of chromosomal aberrations (Evans, 1962). At least 25 cells were analyzed for chromosomal aberrations from one of the positive control cultures. For control of bias, all slides except for the positive controls were coded prior to analysis. Cells with aberrations were recorded on the data sheets by the microscope stage location.

The following factors were taken into account in the evaluation of the chromosomal aberrations data:

The overall chromosomal aberration frequencies.

The percentage of cells with any aberrations.

The percentage of cells with more than one aberration.

Any evidence for increasing amounts of damage with increasing dose, i.e., a positive dose response.

The estimated number of breaks involved in the production of the different types of aberrations which were observed, i.e., complex aberrations may have more significance than simple breaks.

Chromatid and isochromatid gaps, if observed, were noted in the raw data and were tabulated. They were not, however, considered in the evaluation of the ability of the test article to induce chromosomal aberrations since they may not represent true chromosomal breaks and may possibly be induced by toxicity.

A cell classified as "GT" is considered to contain 10 aberrations for statistical purposes but a ">" is also included in the tables for this classification to indicate that it is a minimum number.

Statistical analysis employed the Fisher's Exact Test with an adjustment for multiple comparisons (Sokal and Rohlf, 1981) to compare the percentage of cells with aberrations in each treatment group with the results from the pooled solvent and negative controls (the solvent and negative controls were statistically evaluated for similarity prior to the pooled evaluation). Test

article significance was established where $p \leq 0.01$. All factors as stated previously were taken into account and the final evaluation of the test article was based upon scientific judgement.

DISCUSSION OF RESULTS

Solubility, Stability, and Dose Determination

Solubility of the test article was tested in dimethyl sulfoxide and tetrahydrofuran. Clear colorless solutions were obtained at a concentration of ~100 mg/mL. These solutions were diluted 1:10 in culture medium (MEM) and immiscible globules were obtained. Vortexing and shaking vigorously made the globules smaller which then settled to the bottom of the glass vial before coalescing into larger globules. Similar results were obtained when the test article was diluted directly 1:10 in culture medium. Solubility was tested further in 10% (w/v) solution of Pluronic® F-68 solution. Stable emulsions were produced after this solution was homogenized using a Tissuemizer®. A target top stock of 50.0 mg/mL was selected for testing in these assays. Final concentrations were achieved by a 1:10 dilution of the 50.0 mg/mL stock solution or the serial dilutions prepared from this stock solution in cultures with medium buffered with 25 mM Hepes. A half-log series of concentrations from 0.167 µg/mL through 5010 µg/mL was tested in the SCE assay.

Sister Chromatid Exchange Assay Without Metabolic Activation

Complete cellular toxicity was observed at 167, 501, 1670, and 5010 µg/mL. At 50.1 µg/mL, a slightly unhealthy cell monolayer, floating debris, and a slight reduction in visible mitotic cells. Fluorescent examination of the prepared slides showed cell cycle delay at 16.7 and 50.1 µg/mL and these cultures were reincubated for an additional ~6.5 h. Results were evaluated at 1.67, 5.01, 16.7 (earlier harvest), and 50.1 (later harvest) µg/mL (Table 4-1). A weakly positive response was observed at 50.1 µg/mL. The assay was repeated testing concentrations of 10.0, 25.0, 50.1, 75.1, and 100 µg/mL.

In Trial 2, a dead cell monolayer was observed at 100 µg/mL. An unhealthy cell monolayer, floating dead cells and debris, a severe reduction in visible mitotic cells, and ~60% reduction in the cell monolayer confluence were observed at 75.1 µg/mL. Results were evaluated at 10.0, 25.0, 50.1, and 75.1 µg/mL (Table 4-2). Evaluation of the prepared slides showed severe contamination with yeast in the solvent control culture. The other cultures were free of contamination. Data were not evaluated from this culture and the negative control culture was used for statistical comparison. No significant increase in SCE was observed at the concentrations analyzed. The sensitivity of the cell culture for induction of SCE is shown by the increased frequency of SCE in the cells exposed to the positive control agent. The test article is considered negative for inducing SCE under conditions of nonactivation.

Sister Chromatid Exchange Assay With Metabolic Activation

Complete cellular toxicity was exhibited at 167, 501, 1670, and 5010 µg/mL by the absence of a cell monolayer or the presence of a dead cell monolayer. A ~40% reduction in the cell monolayer confluence was the only evidence of toxicity at 50.1 µg/mL. Fluorescent examination of the prepared slides indicated no cell cycle delay. Results were evaluated at 1.67, 5.01, 16.7, and 50.1 µg/mL (Table 4-3). A weakly positive response was again observed at 50.1 µg/mL. This response was investigated testing concentrations of 10.0, 25.0, 50.1, 75.1, and 100 µg/mL.

In Trial 2, an unhealthy cell monolayer, floating dead cells and debris, ~>90% reduction in the cell monolayer confluence, and a severe reduction in visible mitotic cells were observed at 100 µg/mL. Floating dead cells and debris, reduction in visible mitotic cells, and ~50% reduction in the cell monolayer confluence were observed at 75.1 µg/mL. Results were evaluated at 25.0, 50.1, 75.1, and 100 µg/mL (Table 4-4). A weakly positive increase in SCE was observed only at 100 µg/mL and not at the other doses analyzed. This increase is probably the result of the excessive toxicity exhibited at this dose and not due to the test article induced damage to the DNA. The successful activation of the metabolic system is illustrated by the increased frequency of SCE in the cells induced with the positive control agent. The test article is considered negative for inducing SCE under conditions of metabolic activation.

Chromosomal Aberrations Assay Without Metabolic Activation

Based on the cell cycle kinetics obtained from Trial 1 of the SCE assay, 10 and 20 h harvests were selected for the aberrations assay. This was due to the severe cell cycle delay observed at 50.1 (after 32 h of culture) and the very slight cell cycle delay observed at the subsequent concentration of 16.7 µg/mL. Concentrations of 6.26, 12.5, 18.8, and 25.1 µg/mL were tested with a 10 h harvest and concentrations of 25.0, 50.1, 75.1, and 100 µg/mL were tested with a 20 h harvest. No significant toxicity was visible at any of the doses tested in the 10 h assay. In the 20 h assay, more toxicity was observed at 75.1 µg/mL (~30% reduction in the cell monolayer confluence and a reduction invisible mitotic cells) than at 100 µg/mL (no discernible toxicity). Hence the 20 h assay was repeated testing the same concentrations. In this trial an unhealthy cell monolayer, floating dead cells, a severe reduction in visible mitotic cells, and ~60% reduction in the cell monolayer confluence were observed at 100 µg/mL. Results were analyzed at 25.0, 50.1, 75.1, and 100 µg/mL (results pooled from the replicate cultures are in Table 4-5, and results from individual cultures are in Table 4-6). No significant increase in chromosomally aberrant cells was observed at the concentrations analyzed.

The cell cycle kinetics data for Trial 2 indicated only a slight cell cycle delay, whereas a much larger delay was observed at 50.1 µg/mL in Trial 1. The test article emulsion may not interact uniformly with cell monolayers. Thus, variable toxicity (as occurred at 75.1 and 100 µg/mL in Trial 1 of

the 20 h harvest) and variable cell cycle delay are not unexpected. The 20 h harvest for the aberrations assay remains valid, despite the apparent lack of cell cycle delay, because the test article emulsion was present continuously in the nonactivation assay. Thus, interactions of the test article with cells in S and G phases of the interphase were always occurring, which is necessary and sufficient to allow detection of any induced chromosomal aberrations.

The sensitivity of the cell culture for induction of chromosomal aberrations is shown by the increased frequency of aberrations in the cells exposed to the positive control agent. The test article is considered negative for inducing chromosomal aberrations under nonactivation conditions.

Chromosomal Aberrations Assay With Metabolic Activation

No cell cycle delay was observed at the concentrations analyzed in the SCE assay. A 10 h aberrations assay was selected testing concentrations of 7.51, 11.3, 15.0, 37.5, 75.1, 113, and 150 µg/mL. Complete cellular toxicity was observed in one of the replicate cultures at 150 µg/mL. An unhealthy cell monolayer, floating dead cells and debris, a severe reduction in visible mitotic cells, and ~60% reduction in the cell monolayer confluence were observed in the replicate culture at 150 µg/mL. No significant toxicity was observed in the other cultures. Results were evaluated at 15.0, 37.5, 75.1, 113, and from the surviving culture at 150 µg/mL (results pooled from the replicate cultures are in Table 4-7, and results from individual cultures are in Table 4-8). No significant increase in chromosomally aberrant cells was observed at the doses analyzed. The successful activation of the metabolic system is illustrated by the increased incidence of chromosomally aberrant cells in the cultures induced with CP, the positive control agent. The test article is considered negative for inducing chromosomal aberrations under conditions of metabolic activation.

CONCLUSION

The test article, DCFB, is considered negative for inducing SCE in CHO cells under both the metabolic activation and nonactivation conditions of this assay. It also tested negative for inducing chromosomal aberrations in CHO cells under both the metabolic activation and nonactivation conditions of this assay.

REFERENCES

Bancroft, H. 1957. Introduction to Biostatistics, Hoeber-Harper.

Evans, H.J.. 1962. Chromosomal aberrations produced by ionizing radiation. *International Review of Cytology*, 13:221-321.

Goto, K., S. Maeda, Y. Kano, and T. Sµgiyama 1978. Factors involved in differential Giemsa-staining of sister chromatids. *Chromosoma*, 66:351-359.

Hollander, M , and D.A. Wolfe. 1973. Nonparametric Statistical Methods, John Wiley and Sons.

Perry, P. and S. Wolff. 1074. New Giemsa method for the differential staining of sister chromatids. *Nature*, 251:156-158.

Sokal, R.R., and F.J. Rohlf. 1981. *Biometry*, Ed. 2, W.H. Freeman and Company, New York.

Terasima, T. and Tolmach, L.J. 1961. Changes in X-ray sensitivity of HeLa cells during the division cycle. *Nature*, 190:1210-1211.

TABLE 4-1. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF DCFB (TRIAL 1)

TREATMENT	DOSE µg/mL (µL/mL)	TOTAL CELLS SCORED	# OF CHROMO-SOMES	# OF SCE	SCE/ CHROMO-SOME	SCE/CELL MEAN ± S.E	TIME IN BRDURD (hr)	CELL CYCLE M_1	M_1+	M_2	M_2+	% SCE OVER SOLVENT	CONFLUENCE % SOLVENT CONTROL
CONTROLS													
NEGATIVE: McCoys 5a		50	1030	398	0.39	7.96 ± 0.41	25.6	0.5	3.0	94.0	2.5		
SOLVENT: 10% PLURONIC®	(100)	50	1050	410	0.39	8.20 ± 0.44	25.6	0.5	12.0	87.5			100
POSITIVE: MMC	0.005	20	415	724	1.74	36.20 ± 2.29*	25.6		6.0	94.0		347	88
DCFB	1.67	50	1044	424	0.41	8.48 ± 0.38	25.6	1.5	21.0	77.0	0.5	4	100
	5.01	50	1035	418	0.40	8.36 ± 0.42	25.6	3.0	30.0	67.0		3	100
	16.7	50	1050	418	0.40	8.36 ± 0.40	25.6	5.0	35.0	60.0		2	75
	50.1	50	1048	512	0.49	10.24 ± 0.48*	32.0	17.0	51.5	31.5	0.0	25	63
	167**												25

*Significantly greater than the solvent control, $p<0.05$
**Toxic dose

TABLE 4-2. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF DCFB (TRIAL 2)

TREATMENT	DOSE µg/mL (µL/mL)	TOTAL CELLS SCORED	# OF CHROMO-SOMES	# OF SCE	SCE/ CHROMO-SOME	SCE/CELL MEAN ± SEM	TIME IN BRDURD (hr)	CELL CYCLE M_1	CELL CYCLE M_1+	CELL CYCLE M_2	CELL CYCLE M_2+	% SCE INCREASE OVER NEGATIVE	CONFLUENCE % NEGATIVE CONTROL
CONTROLS													
NEGATIVE McCoys 5a		50	1036	391	0.38	7.82 ± 0.47	25.5		11.0	88.5	0.5		
SOLVENT: 10% PLURONIC® F-68	(10)*												100
POSITIVE: CP	0.500	20	415	733	1.77	36.65 ± 1.50**	25.5		16.0	84.0		372	100
DCFB	10.0	50	1020	370	0.36	7.40 ± 0.33	25.5	0.5	8.5	90.0	1.0		100
	25.0	50	1030	351	0.34	7.02 ± 0.37	25.5		8.0	91.5	0.5		100
	50.1	50	1044	406	0.39	8.12 ± 0.41	25.5		6.0	90.0	4.0	5	100
	75.1	50	1019	393	0.39	7.86 ± 0.47	25.5	0.5	30.0	68.5	1.0	1	38
	100***												6

*Data not included due to yeast contamination
**Significantly greater than the negative control, $p<0.05$
***Toxic dose

TABLE 4-3. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF DCFB (TRIAL 1)

TREATMENT	DOSE µg/mL (µL/mL)	TOTAL CELLS SCORED	# OF CHROMO-SOMES	# OF SCE	SCE/ CHROMO-SOME	SCE/CELL MEAN ± S.E	TIME IN BRDURD (hr)	CELL CYCLE M_1	M_1+	M_2	M_2+	% SCE OVER SOLVENT	CONFLUENCE % SOLVENT CONTROL
CONTROLS													
NEGATIVE:													
McCoys 5a		50	1037	443	0.43	8.86 ± 0.37	25.6		4.0	92.0	4.0		
SOLVENT 10% PLURONIC®	(100)	50	1050	462	0.44	9.24 ± 0.41	25.6		3.0	88.5	8.5		100
POSITIVE: CP	1.50	20	413	760	1.84	38.00 ± 1.24*	25.6		7.5	92.0	0.5	31	100
DCFB	1.67	50	1046	484	0.46	9.68 ± 0.44	25.6		4.0	88.0	8.0	5	100
	5.01	50	1045	524	0.50	10.48 ± 0.55	25.6	0.5	5.0	89.5	5.0	14	100
	16.7	50	1047	492	0.47	9.84 ± 0.37	25.6		2.0	91.0	7.0	7	100
	50.1	50	1042	583	0.56	11.66 ± 0.52*	25.6	1.0	15.0	83.0	1.0	27	63
	167**												6

*Significantly greater than the solvent control, $p<0.01$
**Toxic dose

TABLE 4-4. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF DCFB (TRIAL 2)

TREATMENT	DOSE µg/mL (µL/mL)	TOTAL CELLS SCORED	# OF CHROMOSOMES	# OF SCE	SCE/ CHROMOSOME	SCE/CELL MEAN ± S E	TIME IN BRDURD (hr)	CELL CYCLE M_1	M_1+	M_2	M_2+	% SCE OVER SOLVENT	CONFLUENCE % SOLVENT CONTROL
CONTROLS													
NEGATIVE													
McCoys 5a		50	1048	382	0.36	7.64 ± 0.39	25.5		6.0	92.0	2.0		
SOLVENT 10% PLURONIC® F-68	(10)	50	1043	430	0.41	8.60 ± 0.41	25.5	1.0	9.0	88.5	1.5		100
POSITIVE: CP	1.50	20	415	776	1.87	38.80 ± 1.87*	25.5	0.5	12.0	87.5		354	100
DCFB	25.0	50	1048	473	0.45	9.46 ± 0.47	25.5		10.5	87.0	2.5	9	100
	50.1	50	1044	465	0.45	9.30 ± 0.42	25.5	0.5	11.5	87.5	0.5	8	88
	75.1	50	1049	512	0.49	10.24 ± 0.49	25.5	3.0	28.5	68.0	0.5	18	50
	100	50	1049	528	0.50	10.56 ± 0.46*	25.5	4.0	28.5	65.5	2.0	22	13

*Significantly greater than the solvent control, $p<0.01$

TABLE 4-5. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF DCFB

(Results pooled from duplicate cultures)

			NUMBER AND TYPE OF ABERRATION													NO. OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELL WITH >1 ABERRATION
		SCORED	NOT COMPUTED			SIMPLE			COMPLEX									
			TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
CONTROLS																		
NEGATIVE AND SOLVENT		200	5	1									1			0.01	0.5	0.0
POSITIVE																		
MMC	0.040 µg/mL	25	5	4		1	2		1	2	1	1				0.32	24.0*	4.0
DCFB																		
	25.0 µg/mL	200	11	1									3			0.02	1.5	0.0
	50.1 µg/mL	200	6	2			1									0.01	0.5	0.0
	75.1 µg/mL	200	8	2		1	2									0.02	1.0	0.5
	100 µg/mL	200	3	1												0.00	0.0	0.0

*Significantly greater than the pooled negative and solvent controls, $p<0.01$

TABLE 4-6. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF DCFB

(Results from individual cultures)

		CELLS SCORED	NUMBER AND TYPE OF ABERRATIONS													NO OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATION
			NOT COMPUTED			SIMPLE			COMPLEX									
			TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
CONTROLS																		
NEGATIVE																		
McCoys 5a		100	3										1			0.01	1.0	0.0
SOLVENT:																		
10% PLURONIC® F-68 10.0 µl/mL		100	2	1												0.00	0.0	0.0
POSITIVE:																		
MMC 0.040 µg/mL		25	5	4		1	2		1	2	1	1				0.32	24.0	4.0
DCFB																		
25.0 µg/mL	A	100	3	1									2			0.02	2.0	0.0
	B	100	8										1			0.01	1.0	0.0
50.1 µg/mL	A	100	1	1												0.00	0.0	0.0
	B	100	5	1			1									0.01	1.0	0.0
75.1 µg/mL	A	100	1	1		1	2									0.03	2.0	1.0
	B	100	7	1												0.00	0.0	0.0
100 µg/mL	A	100	3	1												0.00	0.0	0.0
	B	100														0.00	0.0	0.0

TABLE 4-7. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF DCFB

(Results pooled from duplicate cultures)

	CELLS SCORED	NUMBER AND TYPE OF ABERRATION													NO OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATION
		NOT COMPUTED			SIMPLE			COMPLEX									
		TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	P	C			
CONTROLS																	
NEGATIVE AND SOLVENT	200	9	1												0 00	0 0	0 0
POSITIVE:																	
CP 25.0 µg/mL	25	2	3	?		2		1	2	1	1				0 28	20 0*	8.0*
DCFB																	
15 0 µg/mL	200	3	2							1		1			0 01	1 0	0 0
37 5 µg/mL	200	4	2												0 00	0 0	0 0
75.1 µg/mL	200	15	5		2	1									0 02	1 5	0 0
113 µg/mL	200	3	3		1	1									0 01	1 0	0.0
150 µg/mL	100	6	7			1									0 01	1.0	0.0

*Significantly greater than the pooled negative and solvent controls, $p<0.01$

TABLE 4-8. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF DCFB

(Results from individual cultures)

		CELLS SCORED	NUMBER AND TYPE OF ABERRATION													NO OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATION
			NOT COMPUTED			SIMPLE			COMPLEX									
			TG	SG	UC	TB	SB	DM	ID	TR	OR	CR	D	R	CI			
CONTROLS																		
NEGATIVE																		
McCoys 5a		100	6	1												0 00	0 0	0 0
SOLVENT																		
10% PLURONIC® F-68 10 0 µl/mL		100	3													0 00	0 0	0 0
POSITIVE																		
MMC 0.040 µg/mL		25	2	3	2		2		1	2	1	1				0 28	20 0	8 0
DCFB																		
15 0 µg/mL	A	100	1								1		1			0 02	2 0	0 0
	B	100	2	2												0 00	0 0	0 0
37 5 µg/mL	A	100	3	1												0 00	0 0	0 0
	B	100	1	1												0 00	0 0	0 0
75 1 µg/mL	A	100	6			1										0 01	1 0	0 0
	B	100	9	5		1	1									0 02	2 0	0 0
113 µg/mL	A	100	2													0 00	0 0	0 0
	B	100	1	3		1	1									0 02	2 0	0 0
150 µg/mL	A	0																
	B	100	6	7			1									0 01	1 0	0 0

APPENDIX 4-A

DEFINITIONS OF CHROMOSOME ABERRATIONS FOR GIEMSA STAINED CELLS

NOT COMPUTED

TG Chromatid Gap: ("tid gap"). An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks.

SG Chromosome Gap: ("isochromatid gap, IG"). Same as chromatid gap but at the same locus in both sister chromatids.

UC Uncoiled Chromosome: Failure of chromatin packing. Probably not a true aberration.

PP Polyploid cell: A cell containing multiple copies of the haploid number (n) of chromosomes. Only indexed if very common. Not counted in the cells scored for aberrations.

E Endoreduplication: 4n cell in which separation of chromosome pairs has failed. Only indexed if very common. Not counted in the cells scored for aberrations.

SIMPLE

TB Chromatid Break: An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced.

SB Chromosome Break: Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated. This classification now includes the acentric fragment (AF). The AF was different from the SB only in that it was not apparently related to any specific chromosome.

DM "Double Minute" fragment: These are small double dots, some of which are terminal deletions and some interstitial deletions and probably small rings. Their origins are not distinguishable.

COMPLEX

ID Interstitial Deletion:	Length of chromatid "cut out" from midregion of a chromatid resulting in a small fragment or ring lying beside a shortened chromatid or a gap in the chromatid.
TR Triradial:	An exchange between two chromosomes, or one chromosome and an acentric fragment, which results in a three-armed configuration.
QR Quadriradial:	As triradial, but resulting in a four-armed configuration.
CR Complex Rearrangement:	An exchange among more than two chromosomes or fragments which is the result of several breaks.
D Dicentric:	An exchange between two chromosomes which results in a chromosome with two centromeres. This is often associated with an acentric fragment in which case it is classified as DF.
DF	Dicentric with fragment.
TC Tricentric:	An exchange involving three chromosomes and resulting in a chromosome with three centromeres. Often associated with two to three AF. Such exchanges can involve many chromosomes and are named as follows:
QC Quadricentric:	four centromeres, up to four AF
PC Pentacentric:	five centromeres, up to five AF
HC Hexacentric:	six centromeres, up to six AF
R Ring:	A chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as RF.
RC Ring Chromatid:	Single chromatid ring (acentric).
RF	Ring with associated acentric fragment.
CI Chromosome Intrachange:	Exchange within a chromosome; e.g., a ring thatoes not include the entire chromosome.
T Translocation:	Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes. When identifiable, scored as "T" not "2Ab."

AB	Abnormal monocentric chromosome. This is a chromosome whose morphology is abnormal for the karyotype, and often the result of a translocation, pericentric inversion, etc. Classification used if abnormality cannot be ascribed to; e.g., a reciprocal translocation.
OTHER	
GT/>	A cell which contains more than 10 aberrations. A heavily damaged cell should be analyzed to identify the types of aberrations and may not actually have >10, e.g., multiple fragments such as those found associated with a tricentric.

SECTION 5

IN VITRO TRANSFORMATION OF BALB/C-3T3 CELLS WITH AND WITHOUT S9 ACTIVATION OF 1,3-DICHLOROTETRAFLUOROBENZENE

Myhr, B.[a]

ABSTRACT

1,3-Dichlorotetrafluorobenzene (DCFB) was assayed for its ability to induce morphological cell transformation in BALB/c-3T3 cell cultures in the absence and presence of a rat liver S9 metabolic activation system. Six doses ranging from 25 µg/mL to 250 µg/mL were examined as emulsions in EMEM culture medium containing 1% w/v Pluronic® F-68. The toxicity, determined from the clonal survival of ouabain-resistant cells in the presence of a wildtype monolayer, ranged from 92% to 9% without S9 and from essentially 100% at 50 µg/mL (the 25 µg/mL treated cultures were lost) to 11% at 250 µg/mL with S9. The number of transformed foci in the DCFB-treated cultures did not change from the corresponding negative controls. Therefore, DCFB was evaluated as negative for the induction of morphological transformation in BALB/c-3T3 cell cultures.

INTRODUCTION

BALB/c-3T3 mouse cells multiply in culture until a uniform monolayer is achieved and then cease further division (T. Kakunaga, 1973; J.O. Rundell, 1983). These nontransformed cells, if injected into immunosuppressed mice (1×10^7 cells/animal), do not produce neoplastic tumors (Kakunaga, 1973; Rundell et al., 1983; Rundell, 1984.). However, BALB/c-3T3 cells treated *in vitro* with some chemical carcinogens give rise to foci of morphologically altered cells superimposed on the contact-inhibited cell monolayer. If foci picked from cell cultures are grown to larger cell numbers and are injected into immunosuppressed mice, a malignant tumor will be obtained in most cases. Thus, the appearance of foci of altered cells is correlated with malignant transformation.

The ability of BALB/c-3T3 to metabolize test articles from various chemical classes can be enhanced by the addition of an exogenous S9 metabolic activation system to the cultures during the treatment period. However, the standard treatment period of 72 h is reduced to only four hours because of S9 toxicity and the degradation of the NADPH-dependent 450 enzyme system, so this assay modification may not always detect procarcinogens. The procarcinogen dimethylnitrosamine (DMN) does not transform BALB/c-3T3 cells in the absence of S9 (Rundell et al., 1983), but DMN treatments with the S9 activation system usually induce statistically significant increases in the frequency of

[a] Hazleton Laboratories America, Inc., 5516 Nicholson Lane, Kensington, MD 20895

transformed foci (Matthews and Rundell). Similarly, S9-dependent induction of transformed foci by DMN has been reported for another mouse line (Tu et al., 1984).

An appropriate dose range for toxic test articles is selected by a novel method for determining clonal survival under the mass culture conditions of the transformation assay. Normal BALB/c-3T3 cells are sensitive to the cell membrane poison, ouabain, and are quickly killed. However, a mutant BALB/c-3T3 cell line has been established in this laboratory that is ouabain-resistant but otherwise as sensitive to test articles as the parent (wildtype), ouabain-sensitive cells. When a few (eg., 600) ouabain-resistant cells are mixed with a large number of wildtype cells (eg., 7×10^4 cells), the clonal survival of the ouabain-resistant cells can be determined by the addition of 4 mM ouabain to the culture medium after the treatment period. In this manner, the test article toxicity is determined under the same cellular exposure conditions that will occur in the transformation assay mass cultures.

The objective of this assay was to evaluate 1,3-dichlorotetrafluorobenzene (DCFB) for its ability to induce the malignant transformation of cultured BALB/c-3T3 mouse cells in the absence and presence of a rat liver S9 metabolic activation system. Transformation is recognized as a dense, piling up of morphologically altered cells, called a focus, superimposed on a monolayer of contact-inhibited cells (Heidelberger et al., 1983; Rundell, 1984).

MATERIALS AND METHODS

Indicator Cells

Clone 1-13 of BALB/c-3T3 mouse cells was obtained from Dr. T. Kakunaga. A subclone of these cells with a low spontaneous frequency of transformants was used for the transformation assays in this study. Stocks of cells were maintained in liquid nitrogen and have been checked to ensure the absence of mycoplasma contamination. Laboratory cell cultures were grown in Eagle's minimum essential medium (EMEM) supplemented with fetal bovine serum (FBS), L-glut-amine, and antibiotics.

Controls

Negative (Solvent) Control

The negative control (also referred to as the solvent control) was EMEM culture medium containing the emulsifier, Pluronic® F-68, at a concentration of 1% w/v. In addition, for the S9 activation assay, the S9 activation system was added to the medium.

Medium Control

A medium control consisting only of EMEM culture medium or medium containing the S9 activation system was also included with each assay. This control was used to detect any significant effects that might be caused by the 1% Pluronic® F-68 component.

Positive Controls

A known carcinogen, 3-methylcholanthrene (MCA), was used at 2.5 µg/mL as a positive control for the transformation of BALB/c-3T3 cells in the absence of S9.

A second known carcinogen, DMN, was used as the positive control for the transformation of BALB/c-3T3 cells in the presence of the S9 activation system. DMN requires activation by S9 microsomal enzymes to become transforming, and two or more concentrations of DMN within the 1 to 10 µL/mL range were chosen to demonstrate a significant response for at least one concentration of DMN.

Test Material

The test material, DCFB, was a clear, colorless liquid. It was stored at room temperature in its original glass container in a chemical cabinet.

S9 Metabolic Activation System

S9

A 9000 × g supernatant fraction (Ames, 1984) prepared from the livers of Aroclor 1254-induced Sprague-Dawley male rats was purchased commercially and used for this study. The S9 was prescreened and selected for relatively low toxicity to BALB/c-3T3 cells and for the conversion of DMN to toxic substances. The concentration of S9 selected for the assay was 40 µL/mL, which corresponded to 1.4 mg S9 protein/mL in the treatment medium.

CORE

The S9 activation system included a NADPH regenerating system (CORE) composed of NADP and isocitric acid in the presence of S9. The final concentrations of the CORE components in the treatment medium were approximately 236 µg/mL of NADP (sodium salt) and approximately 1552 µg/mL of isocitrate. The CORE solution was prepared fresh as a 20X stock solution in culture medium and was then combined with S9 to give a 10X S9 mix. The mix was held on ice until used in the assay.

EXPERIMENTAL DESIGN

Test Material Preparation

In order to achieve as uniform an exposure as possible for cell monolayers bathed in culture medium, Pluronic® F-68 was investigated as an emulsifying agent for the test material, DCFB. Pluronic® F-68 is a polyalcohol that is frequently used in cell cultures due to its low toxicity and ability to lower surface tension. When a preparation of 50 mg/mL of DCFB in 10% w/v Pluronic® F-68 in deionized water was vigorously agitated for approximately one minute with a Tissumizer® at a

setting of 40, a stable, white emulsion was formed. The DCFB remained dispersed into tiny droplets after diluting 1:10 into culture medium and did not coalesce upon sitting.

A fresh emulsion of DCFB was prepared in 10% w/v Pluronic® F-68 in deionized water for each dose rangefinding study and the transformation assays. A Tissumizer® was used at a setting of 40-50 for 30 s to 1 min to prepare the emulsions. The initial concentration of DCFB ranged from 10 mg/mL to 50 mg/mL, and lower concentrations were prepared by diluting the emulsions into 10% w/v Pluronic® F-68. The emulsions were mixed well by vortex before preparing the treatment media. The treatment media were prepared as 1:10 dilutions of the emulsions into EMEM culture medium so that the Pluronic® F-68 content was diluted to 1% w/v

Preliminary Dose Rangefinding

Glass culture bottles with approximately 60 cm^2 of surface area were seeded concurrently with approximately 600 ouabain-resistant 3T3 cells and 7×10^4 wildtype cells. The following day, one culture was exposed to each of nine doses with and without S9, starting at 5000 µg/mL and diluting in 2-fold steps. Two solvent control cultures containing 1% w/v Pluronic® F-68 in EMEM culture medium were prepared for both test conditions. After an exposure period of approximately four hours in the presence of the S9 activation system or approximately 72 h without S9, the cells were washed with a physiological solution and refed with EMEM culture medium containing 4 mM ouabain. The cultures were refed with medium containing 4 mM ouabain four to five days later. Surviving colonies were terminated 7-10 days after the treatment periods, stained with Giemsa, and counted manually.

A relative survival for each treatment condition was obtained by comparing the number of surviving colonies to the average colony count for the solvent control cultures. This survival information was used to select adjusted dose ranges for additional dose rangefinding studies.

A total of four dose rangefinding studies were conducted before a stable response was obtained over an appropriate dose range. In the last trial, eight doses ranging from 300 to 10 µg/mL were used with and without S9. Cultures which were treated with selected doses but not with ouabain were included to check on the mass culture responses; these cultures were terminated four days after treatment initiation, when it was indicated that an appropriate span of toxicity was achieved. Thus, the rangefinding cultures without S9 were refed ouabain medium four days after the treatment period and were terminated and stained seven days later. The cultures with S9 were refed ouabain medium seven days after the treatment period and were terminated seven days later. The survival information from this trial was used to select six doses for the transformation assay that would span a survival range of approximately 10% to 100%.

Transformation Assay

The transformation assay procedure was adapted from that reported by Kakunaga (1984). Glass culture bottles having a monolayer growth area of approximately 60 cm^2 were used. Each bottle was seeded with approximately 7×10^4 cells for the transformation assay and 7×10^4 cells plus approximately 600 ouabain-resistant cells for the concurrent clonal survival assay. On the day after seeding, nine cultures were exposed to each selected treatment with DCFB and the positive controls. Eighteen cultures were used as solvent controls for each of the test conditions (with and without the S9 activation system). In addition, nine cultures were used as EMEM medium controls without Pluronic® F-68 for both test conditions. One mixed culture of wildtype and ouabain-resistant cells was exposed to each treatment and control condition.

The treatments were conducted at 37 ± 1°C for approximately four hours with the S9 activation system and approximately 72 h without S9. The treatments were initiated by adding 1.0 mL of a 10X suspension of DCFB or solution of positive control to each culture containing 9 mL of culture medium (with or without the S9 activation system). After treatment, all cultures were washed with Hanks' balanced salt solution. The transformation assay cultures were refed with fresh EMEM culture medium, and the incubation was continued for approximately four weeks with refeeding twice a week. The clonal survival cultures were refed with medium containing 4 mM ouabain. The cultures without S9 were refed with 4 mM ouabain eight days later and were terminated 11 days after treatment. The S9 activation cultures were refed with four mM ouabain 11 days after treatment and terminated 14 days after treatment.

The cultures were terminated by fixing the cells with methanol and staining with 10% Giemsa in tap water. Stained cultures were examined by eye and by microscope to determine the number of foci of transformed cells and the colony survival. The transformation assay cultures were coded with random numbers prior to evaluation for foci.

Evaluation of Transformed Foci

At the end of the incubation period, cultures of cells with a normal phenotype yield a uniformly stained monolayer of round, contact-inhibited cells. Transformed cells form a multi-layered mass of cells, or focus, that stains deeply and is superimposed on the surrounding monolayer of cells (Kakunaga, 1973; Rundell, 1984). The foci are variable in size and exhibit several variations in morphological features. Many scored foci consist of a dense piling-up of cells with a random, criss-cross orientation of fibroblastic cells at the periphery of the focus and extensive invasiveness into the contiguous monolayer. Other scored foci are composed of more rounded cells with little criss-crossing at the periphery but with necrosis at the center caused by the dense piling-up of a large

number of cells. A third variation is a focus without the necrotic center and large number of cells but which exhibits the criss-cross pattern of overlapping cells throughout most of the colony.

Some densely stained areas are not scored as transformed foci because the random orientation of fibroblastic cells is not observed. Microscopic examination is routinely employed for scoring and for the final judgement of the transformed character of each focus.

All foci that exhibited the transformed characteristics were scored. In the raw data, a record of focus size was maintained by scoring foci greater than four mm in diameter as + + + and those of two to four mm in diameter as + +. No significance is currently attached to this categorization. The sum of all scored foci (+ + + and + +) was reported for each culture and was used for the assay analysis.

Assay Acceptance Criteria

The clonal survival assay conducted simultaneously with the transformation assay was considered acceptable for evaluation of the test results by meeting the following three criteria.

1. The negative (solvent) control cultures must have macroscopically visible BALB/c-3T3 cell colonies representing a cloning efficiency of 15% or greater.
2. At least one of the test material treatments resulted in ≥ 50% cell survival.
3. A cytotoxic dose response was obtained for the test material treatments, unless the test material was nontoxic at 10 mg/mL or its solubility limit in culture medium was exceeded.

The transformation assay was considered acceptable for evaluation of test results by meeting the following five criteria.

1. Negative (solvent) control, positive control, and test material treatments resulted in contiguous monolayers of cells to be evaluated.
2. Negative control spontaneous frequencies of transformation did not exceed an average of approximately two foci per culture.
3. At least one of the positive control treatments resulted in an average number of foci per culture vessel that was significantly different from the negative control at the 99% confidence level ($p \leq 0.01$).
4. A minimum number of six culture vessels per test condition was available for analysis.
5. A minimum number of three treatment levels of the test material was available for analysis.

In addition, the cytotoxicity dose-related data from the preliminary and simultaneous clonal survival assays should be qualitatively similar over a comparable range of test chemical treatments.

Assay Evaluation Criteria

The appearance of transformed foci usually occurs as a general increase in foci for all cultures exposed to a transforming dose. However, large numbers of foci may appear at random in one or more culture vessels in a treatment set, resulting in skewing of the mean number of foci in that set.

This skewing could be caused by factors such as mechanical disruption and respreading of transformed foci cells or a culture-conditioning effect caused by the early appearance of a focus. The appearance of occasional dishes with numerous foci is a random process and occurs in both treated and control cell cultures. In our laboratory, we have utilized a $\log_{10}$ mathematical transformation to handle this non-normal distribution of BALB/c-3T3 cell transformed foci data (Rundell et al., 1983); however, other mathematical models have also been proposed (Whorton et al., 1982). After performing a $\log_{10}$ transformation of the data, Bailey's modification of the Student's t-test (Bailey, 1959) was used to evaluate positive control and test chemical treatment transforming activity for significant differences from the negative control. The possible spectrum of responses was routinely subdivided into three levels for the evaluation of each treatment.

Evaluation of Individual Treatments

Strong positive response = $p \leq 0.01$

Weak positive response = $p \leq 0.05$

Negative response = $p > 0.05$

The results of each treatment condition were evaluated in relation to the observed activities of model compounds, and scientific judgement was exercised in the evaluation of each test material. In general, a response at only one dose attaining a 95% confidence level is not considered sufficient evidence for activity in this assay. However, responses at two or more treatment levels attaining the 95% confidence level and exhibiting evidence of a dose relationship are considered as evidence for transformation. Responses achieving the 99% confidence level for one or more test material treatments are usually considered sufficient for a positive evaluation.

RESULTS AND DISCUSSION

Clonal Survival

Some difficulty was encountered in establishing the appropriate dose range for the transformation assays. The initial dose rangefinding experiment showed lethality from 5000 to 1250 µg/mL and a sudden increase in survival to 34% with S9 and 47% without S9 when the dose was decreased by the next step to 625 µg/mL. Subsequently, two additional experiments showed that 200 µg/mL and higher doses were highly toxic or lethal with or without S9. The third data record was incomplete regarding the treatment period and colony growth. Thus, a fourth trial was required to define the toxicity curve and determine if the toxicity would stabilize in a lower dose range than indicated by the first trial. The results of this last trial are given in Tables 5-1 and 5-2.

With or without S9, DCFB was found to be essentially lethal at 300 µg/mL and to have little or no toxicity at 50 µg/mL (Tables 5-1 and 5-2). This toxicity range was consistent with the observations

of the second and third trials, so the results indicated a stable response range for the selection of doses for the transformation assay. Five doses were chosen at 50 µg/mL increments from 50 to 250 µg/mL, and a sixth dose (25 µg/mL) was also included to help define the variation in response for nontoxic treatments.

Transformation Assay Without S9

Table 5-3 summarizes the results obtained for DCFB in the transformation assay without S9. For treatments with 25 to 250 µg/ml (92% survival to 9% survival), the frequency of transformed foci remained equivalent to the solvent and medium controls. In contrast, the MCA positive control induced a significant increase in focus formation. Thus, no evidence was obtained for transforming activity by DCFB without S9 in BALB/c-3T3 cell cultures.

Transformation Assay with S9

Table 5-4 summarizes the results obtained for DCFB in the transformation assay with S9. The frequency of transformed foci in the cultures exposed to DCFB remained comparable to the solvent and medium negative controls. An apparent increase for the relatively nontoxic treatment with 100 µg/ml was due to one culture which contained 5 foci. The higher doses of 150, 200 and 250 µg/ml spanned the entire survival curve (105% to 11% survival) and caused no increases in foci over the solvent control. Both of the DMN positive controls clearly induced foci. The addition of S9 activation conditions therefore did not confer any transforming activity to DCFB.

CONCLUSION

The test material, DCFB, was evaluated in the absence and presence of a rat liver S9 metabolic activation system within a dose range of 25 to 250 µg/ml. A wide range of toxic response was obtained, but no significant changes in the frequency of transformed foci were observed. Therefore, under the conditions of this study, DCFB was evaluated as negative for transforming BALB/c-3T3 cells in culture.

REFERENCES

Ames, B.N., W.E. Durston, E. Yamasaki, and F.D. Lee. 1984. Carcinogens are mutagens; a simple test system combining liver homogenates for activation and bacteria for detection, *Proc. Natl. Acad. Sci.* USA, 78:6858-6862.

Bailey, Normal T.J. 1959. *Statistical Methods in Biology*, Wiley and Sons, Inc., NY, p. 50.

Heidelberger, C., A.E. Freeman,R.J. Pienta, A. Sivak, J.S. Bertram, B.C. Casto, V.C. Dunkel, M.W. Francis, T. Kakunaga, J.B. Little, and L.M. Schechtman. 1983. Cell transformation by chemical agents - a review and analysis of the literature. *Mutat. Res.* 114:283-385.

Kakunaga, T. 1973. A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB/3T3. *Int. J. Cancer* 12:463-473.

Matthews, E.J. and J.O. Rundell. S9-mediated activation of procarcinogens to cytotoxic, mutagenic, and transforming intermediates for BALB/c-3T3 cells. Prepared manuscript.

Rundell, J.O., M. Guntakatta, and E.J. Matthews. 1983. Criterion development for the application of BALB/c-3T3 cells to routine testing for chemical carcinogenic potential. In: M.D. Waters, et al. (eds.) *Short-term Bioassays in the Analysis of Complex Environmental Mixtures III*. Plenum Publishing Co., NY, pp. 309-327.

Rundell, J.O. 1984. *In vitro* cell transformation, an overview. In: J.F. Douglas (ed.) *Carcinogenesis and Mutagenesis Testing*. Humana Press, pp. 39-61.

Rundell, J.O. 1984. *In vitro* transformation assays using mouse embryo cell lines: BALB/c-3T3 cells. In: J.F. Douglas (ed.) *Carcinogenesis and Mutagenesis Testing*. Humana Press, pp. 279-285.

Tu, A.S., P.A. Green, and A. Sivak. 1984. Comparison of primary hepatocytes and S9 metabolic activation systems for the C3H10T½ cell transformation assay. *Carcinogenesis*, 5:1431-1436.

Whorton, E.B., J.B. Ward, and D.L. Morris. 1982. Experimental design and statistical analysis considerations for *in vitro* mammalian cell transformation assays with BALB/c-3T3 cells. *Environ. Mutagen*. 4:595-603.

TABLE 5-1. CYTOTOXIC ACTIVITY OF 1,3-DICHLOROTETRAFLUOROBENZENE IN THE PRELIMINARY CLONAL SURVIVAL ASSAY WITHOUT S9 ACTIVATION (TRIAL 4)

TREATMENT CONDITION	COLONIES/CULTURE	RELATIVE CELL SURVIVAL %
Solvent Control[a]	217	100.0
DCFB µg/mL:		
10	189	87.1
25	175	80.6
50	183	84.3
100	51	23.5
150	94	43.3
200	62	28.6
250	15	6.9
300	3	1.4

[a] EMEM culture medium containing 1% Pluronic® F-68

[b] Colony survival relative to the solvent control, execpt for the positive control, which is relative to the medium control

[c] The mean transforming activity is expressed as the anti-log of the log10 mean minus one

[d] Solvent Control: EMEM culture medium containing 1% Pluronic® F-68 Average of two cultures (176,204)

TABLE 5-2. CYTOTOXIC ACTIVITY OF 1,3-DICHLOROTETRAFLUOROBENZENE IN THE PRELIMINARY CLONAL SURVIVAL ASSAY WITH S9 ACTIVATION (TRIAL 4)

TREATMENT CONDITION	COLONIES/CULTURE	RELATIVE CELL SURVIVAL %
Solvent Control[a]	185	100.0
DCFB µg/mL:		
10	173	93.5
25	168	90.8
50	204	110.3
100	107	57.8
150	52	28.1
200	46	24.9
250	23	12.4
300	0	0

[a] EMEM culture medium containing 1% Pluronic® F-68

TABLE 5-3. TRANSFORMING ACTIVITY OF 1,3-DICHLOROTETRAFLUOROBENZENE ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITHOUT S9 ACTIVATION

TREATMENT CONDITION	COLONY COUNT[a]	RELATIVE SURVIVAL[b] (%)	FOCUS DATA			TRANSFORMING[c] ACTIVITY
			TOTAL CULTURES	TOTAL FOCI	AVERAGE FOCI/ CULTURE	MEAN FOCI/ CULTURE
Solvent Control[d]	190	100.0	14	5	0.36	0.28
Positive Control[e]	50	35.0	9	330	36.7	31.3**
Medium Control[f]	143	75.3	9	7	0.78	0.66
DCFB µg/mL:						
25	175	92.1	9	2	0.22	0.13
50	141	74.2	9	6	0.67	0.54
100	143	75.3	8	3	0.38	0.30
150	119	62.6	9	6	0.67	0.59
200	85	44.7	9	4	0.44	0.32
250	17	8.9	9	3	0.33	0.17

[a] Clonal survival assay performed concurrently with the transformation assay
[b] Colony survival relative to the solvent control, except for the positive control, which is relative to the medium control
[c] The mean transformation activity is expressed as the anti-log of the $\log_{10}$ mean minus one
[d] Solvent Control: EMEM culture medium containing 1% Pluronic® F68. Average of two cultures (176, 204)
[e] Positive Control: The positive control treatment was 3-methylcholanthrene at 2.5 µg/mL
[f] Medium Control: EMEM culture medium without Pluronic® F-68
**$p \leq 0.01$

TABLE 5-4. TRANSFORMING ACTIVITY OF 1,3-DICHLOROTETRAFLUOROBENZENE ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITH S9 ACTIVATION

TREATMENT CONDITION	COLONY COUNT[a]	RELATIVE SURVIVAL[b] (%)	FOCUS DATA			TRANSFORMING[c] ACTIVITY
			TOTAL CULTURES	TOTAL FOCI	AVERAGE FOCI/ CULTURE	MEAN FOCI/ CULTURE
Solvent Control[d]	158.5	100.0	16	8	0.50	0.36
Positive Control[e]						
DMN, 1 µL/mL	137	84.6	9	18	2.00	1.08*
DMN, 2 µL/mL	87	53.7	9	58	6.44	5.67**
Medium Control[f]	162	102.2	9	2	0.22	0.13
DCFB µg/mL:						
25	C	---	C	---	---	---
50	166	104.7	9	4	0.44	0.32
100	131	82.6	9	9	1.00	0.66
150	166	104.7	9	2	0.22	0.17
200	108	68.1	7	3	0.43	0.29
250	17	10.7	9	2	0.22	0.17

[a] Clonal survival assay performed concurrently with the transformation assay
[b] Colony survival relative to the solvent control, except for the positive controls, which are relative to the medium control
[c] The mean transforming activity is expressed as the anti-log of the log10 mean minus one
[d] Solvent Control: EMEM culture medium containing 1% Pluronic® F-68. Average of two cultures (178, 139)
[e] Positive Control: The positive control treatment was dimethylnitrosamine (DMN) at the concentrations shown
[f] Medium Control: EMEM culture medium without Pluronic® F-68
C = Contaminated
*$p \leq 0.05$
**$p \leq 0.01$

SECTION 6

IN VIVO/IN VITRO RAT PRIMARY HEPATOCYTE UNSCHEDULED DNA SYNTHESIS FOLLOWING TREATMENT WITH 1,3-DICHLOROTETRAFLUOROBENZENE

Cifone, M.A.[a]

ABSTRACT

In the *In Vivo/In Vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay, the test material, 1,3-dichlorotetrafluorobenzene (DCFB), did not induce significant changes in the nuclear labeling of rat hepatocytes over a dose range of approximately 5000 to 625 mg/kg. Three male F344 rats were treated by oral gavage at each of four doses with the test material solubilized in corn oil. At approximately 4.0 h after treatment with the test material, primary hepatocyte cultures were prepared. Viabilities of the hepatocytes obtained ranged from 82.9% to 96.7%. After attachment, the cultures were incubated with 10 µCi/mL ^{3}H-thymidine for four hours. The cultures were prepared for analysis of nuclear labeling 16.4 to 17.9 h after removal of the radioactivity and addition of 0.17 mM thymidine. None of the criteria used to indicate UDS was approached by the treatments and no dose-related response was observed. The test material, DCFB, was therefore evaluated as inactive in the *In Vivo/In Vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay.

INTRODUCTION

This assay is designed to measure unscheduled DNA synthesis (UDS) in rat liver cells (hepatocytes) using the autoradiographic technique described by Williams (1980). Hepatocytes were isolated from the livers of rats exposed *in vivo* to the test article. Only a small percentage of the cells enter S-phase (replicative DNA synthesis) during the exposure period, so the incorporation of ^{3}H-thymidine (^{3}HTdr) into DNA during *in vitro* culturing, as analyzed by autoradiography, may be used as a measure of the repair of DNA damage caused by treatment with the test article. This UDS measurement of DNA repair appears to correlate well with known mutagenic or carcinogenic activities of chemicals (Williams, 1977).

The objective of this assay was to detect DNA damage caused by the test material, 1,3-dichlorotetrafluorobenzene (DCFB), or an active metabolite, by measuring UDS induced in rat primary hepatocytes *in vivo*. The existence and degree of DNA damage was inferred from an increase in net nuclear grain counts in hepatocytes obtained from treated animals when compared to those

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from untreated animals. The types of DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including ^{3}HTdr) into DNA.

MATERIALS AND METHODS

Indicator Cells

The indicator cells for this assay were hepatocytes obtained from adult male Fisher 344 rats (CDF) weighing 190.6 to 211.8 grams, purchased from Charles River Breeding Laboratories, Incorporated. The animals scheduled for this assay were fed Purina Certified Rodent Chow (Formula 5002) and water *ad libitum*. Animals were quarantined a minimum of five days prior to random assignment to the study and identification by ear tag.

Animals were anesthetized prior to surgery using about 60 mg sodium pentobarbitol (V-Pento)/kg and were exanguinated during the liver perfusion.

The cells were obtained by perfusion of the livers *in situ* with a collagenase solution. Monolayer cultures were established in culture dishes and were used the same day for analysis of the UDS activity. All cultures were maintained as monolayers at about 37°C in a humidified atmosphere containing approximately 5% CO_2.

Media

The cell cultures were established in Williams' Medium E supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 µg/mL streptomycin sulfate, and 150 µg/mL gentamicin (WME +). WME + without serum is referred to as WMEI. After the establishment period, cultures were refed with WMEI containing 10 µCi/mL ^{3}HTdr, 40 to 60 Ci/mMole (WME-treat).

Control Compounds

The test article was solubilized in Mazola 100% pure corn oil (Lot No. 021489). A solvent negative control consisting of three rats treated by oral gavage with corn oil was performed. Dosing volumes did not exceed 5.2 mL/kg (target of 5 mL/kg ± 10%).

The positive control compound is known to induce UDS *in vivo* in rat hepatocytes. Dimethylnitrosamine (DMN, CAS #62-75-9) at approximately 10 mg/kg was used. Three rats were treated by intraperitoneal injection.

Test Material

The test material, DCFB, is a clear colorless liquid. It was stored in a chemical cabinet at room temperature in its original glass container.

Dosing Procedure

For the UDS assay, rats were treated by oral gavage with the test article solubilized in corn oil. The total volume of the test article solution administered did not exceed 5.2 mL/kg (target of 5 mL/kg ± 10%). Fresh preparations of test article in solvent were used for any testing purpose.

Dose Selection and Perfusion Time

For the UDS assay, the highest dose selected was 5000 mg/kg . Three additional doses of test material were prepared a minimum of three animals per dose were treated. One timepoint for UDS was performed at 4.0 to 4.1 h after the administration of a single dose of the test article by oral gavage.

UDS Assay

This assay was based on the procedures in rats described by Williams (1980), Mirsalis, Tyson and Butterworth (1982) and Butterworth et al. (1987). The hepatocytes were obtained by perfusion of livers *in situ* for about four minutes with Hanks' balanced salts (Ca^{++} and Mg^{++} free) containing 0.5 mM ethyleneglycol-bis (-aminoethyl ether)-N, N-tetraacetic acid (EGTA), and HEPES buffer at pH 7.2. Then WMEI containing 50-100 units/mL of collagenase was perfused through the liver for 10 - 11 min. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WMEI culture medium and collagenase. The suspended tissue and cells were allowed to settle to remove cell clumps and debris. The cell suspension was centrifuged and the cell pellet resuspended in WME +. After obtaining a viable cell count, a series of 35-mm culture dishes (at least six per animal containing a 25-mm round, plastic coverslip and at least two per animal to assess attachment efficiency) was inoculated for each animal with approximately 0.5×10^6 viable cells in 3 mL of WME + per dish.

An attachment period of 1.5 to 1.8 h at 37°C in a humidified atmosphere containing 5% CO_2 was used to establish the cell cultures. Unattached cells were then removed and the cultures were refed with 2.5 mL WME-treat. Three of the replicate cultures from each animal were used for the UDS assay; two of the replicates were used to assess attachment. Any remaining cultures were kept for analysis in the event of technical problems with autoradiography.

Attachment efficiency was determined for two cultures from each animal using trypan blue dye exclusion and *in situ* analysis.

After a labeling period of four hours labeled cell cultures were refed with WMEI containing 0.17 mM thymidine and returned to the incubator for 16.4 to 17.9 h. The thymidine concentration was slightly lower than stated in the protocol due to technical error but the difference was small and did not affect the assay. Nuclei were then swollen by addition of 1% sodium citrate to the coverslips

(containing the cell monolayers) for 10 min. The cells were next fixed in three changes of acetic acid:ethanol (1:3) and dried for at least 24 h. The fixed coverslips were mounted on glass slides, dipped in Kodak NTB2 emulsion, and dried. The emulsion coated slides were stored for eight days at 4°C in light-tight boxes containing Drierite. The emulsions were then developed in D19, fixed, and stained with Williams' modified hematoxylin and eosin procedure.

The cells were examined microscopically at approximately 1500 x magnification under oil immersion and the field was displayed on the video screen of an automatic counter. UDS was measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (background count). This value is referred to as the net nuclear grain count. The coverslips were coded to prevent bias in grain counting.

The net nuclear grain count was determined for fifty randomly selected cells on each coverslip (three coverslips per animal) unless otherwise indicated. Only nuclei with normal morphologies were scored, and any occasional nuclei blackened by grains too numerous to count were excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. The mean net nuclear grain count was determined from the triplicate coverslips (150 total nuclei) for each animal and each treatment condition.

Occasionally, a coverslip is recounted at a later date by a different technician. Since a different cell population is generally scored, the average count for fifty cells is used in the calculation of the mean for the triplicate treatment.

Assay Acceptance Criteria

An assay normally will be considered acceptable for evaluation of the test results only if all of the criteria listed below are satisfied. This listing may not encompass all test situations, so the study director must exercise scientific judgment in modifying the criteria or considering other causes that might affect assay reliability and acceptance.

1. The viability of the hepatocytes collected from the perfusion process normally exceeds 70%. A variety of factors can affect cell yield and viability, so values below 70% are not uncommon nor necessarily detrimental. Toxicity of treatment with test article may be reflected in perfusion viability, therefore no lower limit will be set.

2. The viability of the monolayer cell cultures used for the assay treatments must be 70% or greater. Normally, the viability of attached cells is about 85%.

3. The positive control is used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS. For test materials causing weak or no UDS activity, the average response to the positive control treatments must exceed both

criteria used to indicate UDS. For test materials clearly causing a dose-related UDS activity, an assay will be acceptable in the absence of a positive control lost for technical reasons. Historical control values for the positive control are NNG = 26.65 ± 7.69 (range, 15.47 to 37.25) and % ≥ 5 grains per nucleus = 93.1% ± 6.2% (range, 83.3% to 100.0%). Historical control values for the negative control are NNG = -0.81 ± 1.18 (range, -3.27 to 0.47) and % ≥ 5 grains per nucleus = 0.23% ± 0.35% (range, 0% to 0.7%).

4. Grain count data obtained per animal is acceptable as part of the evaluation if obtained from two replicate cultures and at least 50 nuclei per culture. Grain count data should be available from two of the three animals treated.

5. A minimum of three doses will be analyzed for nuclear grain counts. Repeat trials need only augment the number of analyzed doses in the first trial to achieve a total of three concentrations, but must include at least one dose previously assayed as acceptable.

Assay Evaluation Criteria

Several criteria have been established which, if met, provide a basis for evaluation of a test material as active in the UDS assay. The criteria for a positive response are based on a statistical analysis of the historical data as described by Casciano and Gaylor (1983). The test material is considered active in the UDS assay at doses that cause:

1. An increase in the mean net nuclear grain count to at least five grains per nucleus above the concurrent negative control value, leading to a positive number

2. An increase in the percent of nuclei having five or more net grains such that the percentage of these nuclei in test cultures is 10% above the percentage observed in the negative control cultures.

Generally, if the first condition is satisfied, the second will also be met. However, satisfaction of only one condition can also indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns, and weak agents may strongly affect only a minority of the cells. Therefore, both of the above conditions are considered in an evaluation. If the vehicle control shows an average less than -5.00 or more than 1.00 grains per nucleus, the assay will normally be considered invalid. The test material is considered inactive in this assay if:

1. The mean net nuclear grain counts for all dosed groups is less than 1.0 net nuclear grain count above the concurrent negative control value.

2. The percent of nuclei with five or more net grains does not increase more than 2% above the concurrent negative control.

When results are neither clearly positive nor clearly negative, the presence of a dose response, the frequency distribution of cellular responses, and the reproducibility of data among slides is

considered; the test article is then classified as "negative", "weak positive" or "equivocal". Groups in which one out of three animals show increases will be decided on a case by case basis depending on the level of activity in cells from the active animal, the level of activity in cells from the inactive animals and the presence or absence of activity in surrounding groups.

The positive control nuclear labeling is not used as a reference point to estimate mutagenic or carcinogenic risk associated with the UDS activity of the test material. UDS elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen. Some forms of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive controls are used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.

RESULTS AND DISCUSSION

The test article, DCFB, was solubilized in corn oil at concentrations ranging from 1003 to 126 mg/mL. All dosing stocks were prepared just prior to use. The test material appeared to form a clear solution in the solvent. Three rats per dose were treated with about 5000, 2500, 1250 and 625 mg/kg of the test material in volumes which did not exceed about 5.0 mL/kg.

Perfusions were initiated 4.0 to 4.1 h after administration of a single dose of the test article by oral gavage. The hepatocytes collected for the UDS assay ranged in viability (determined by trypan blue exclusion) from 82.9 to 96.7% of the total cells collected in the perfusate (Table 6-1). The attachment efficiency varied from 66.9 to 99.1% and the viability of the attached cells was very good, ranging from 88.4 to 96.9%.

The minimum criteria for a UDS response in this assay were determined by comparison to the averages of the concurrent negative control treatments. A positive response consisted of mean net nuclear grain counts exceeding 4.29 or at least 12.7% of the nuclei containing five or more grains. None of the treatments with the test material samples caused nuclear labeling significantly different from the negative control (see Table 6-2). Furthermore, no dose-related trend was evident. In contrast, the DMN treatments induced large increases in nuclear labeling that greatly exceeded both criteria used to indicate UDS. Since the positive control animals were responsive to DMN, the test results were considered to provide conclusive evidence for the lack of UDS induction by the test material samples.

Heavily-labeled nuclei (blackened with numerous grains) represent cells undergoing DNA replication as opposed to DNA repair. The number present in the negative controls of this study was low and did not interfere with the assay although a slight elevation in S-phase cells was observed with

some of the chemical treatments (see Table 6-2). Only 30 cells (0.67%) among the 4,500 negative control cells screened were heavily labeled.

CONCLUSION

The test material, DCFB, did not induce significant changes in the nuclear labeling of rat primary hepatocytes for a dose range of about 5000 to 625 mg/kg. DCFB was therefore evaluated as inactive in the *In Vivo/In Vitro* Rat Primary Hepatocyte UDS Assay.

REFERENCES

Mirsalis, J.C., C.K. Tyson, and B.E. Butterworth. 1982. Detection of genotoxic carcinogens in the *in vivo - in vitro* hepatocyte DNA repair assay. *Environ. Mutagenesis*, 4:553-562.

Williams, G.M. 1980. The detection of chemical mutagens-carcinogens by DNA repair and mutagenesis in liver cultures, In: F. de Serres and A. Hollaender, (eds.) *Chemical Mutagens*, Vol. 6. , Plenum Press, NY, pp. 61-79.

Williams, G.M. 1977. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture. *Cancer Res.*, 37:1845-1851.

Butterworth, B.E., J. Ashby, E Bermudez, D. Casciano, J. Mirsalis, G. Probst, and G. Williams. 1987. A protocol and guide for the *in vivo* rat hepatocyte DNA-repair assay. *Mutation Res.*, 189:123-133.

Casciano, D.A. and D.W. Gaylor 1983. Statistical criteria for evaluating chemicals as positive or negative in the hepatocytes DNA repair assay. *Mutation Research*, 122:81-86.

TABLE 6-1. CULTURE DATA SUMMARY FOR HEPATOCYTES FROM RATS TREATED WITH 1,3-DICHLOROTETRAFLUOROBENZENE

Target Dose (mg/kg)	Animal Number	Perfusion[a] Viability(%)	Attachment[b] Efficiency(%)	(%) Viability[b]
VC	3239	96.5	81.8	96.9
VC	3240	96.7	79.3	94.0
VC	3248	91.6	94.1	93.7
625	3242	83.2	87.1	94.8
625	3244	91.8	74.2	90.6
625	3268	92.5	71.9	91.4
1250	3246	94.7	70.7	92.6
1250	3247	90.2	75.0	88.4
1250	3269	91.4	77.8	89.8
2500	3243	83.0	75.8	94.2
2500	3245	88.6	80.3	93.0
2500	3250	96.7	77.8	95.7
5000	3238	85.8	99.1	90.3
5000	3241	82.9	76.1	91.7
5000	3267	94.0	66.9	89.8
DMN	3249	88.5	88.0	95.7
DMN	3266	85.8	74.8	94.6
DMN	3270	88.6	96.0	96.0

[a] At time of hepatocyte collection (determined by trypan blue exclusion)
[b] Two culture dishes per animal were counted *in situ* after the attachment period using dilute trypan blue to determine viability
DMN = Positive control, dimethylnitrosamine, 10 mg/kg
VC = Vehicle control, 5 mL/kg of corn oil

TABLE 6-2. UDS DATA SUMMARY FOR HEPATOCYTES FROM RATS TREATED WITH 1,3-DICHLOROTETRAFLUOROBENZENE

Target Dose (mg/kg)	Animal Number	Mean (± S.D.) Net Nuclear Grains[a]	Mean Cyto Grains[b]	% Nuclei with ≥ 5 Net Grains	% S[c]
VC	3239	- 1.38 (± 0.31)	9.89	0.7	0.80
VC	3240	- 0.18 (± 0.33)	6.53	4.0	0.60
VC	3248	- 0.57 (± 0.34)	8.97	3.3	0.60
625	3242	- 0.49 (± 0.22)	5.76	1.3	0.73
625	3244	- 0.31 (± 0.26)	7.05	3.3	1.53
625	3268	- 0.67 (± 0.09)	6.80	2.0	0.87
1250	3246	- 0.15 (± 0.12)	6.41	4.0	1.07
1250	3247	0.09 (± 0.18)	5.87	2.0	1.13
1250	3269	- 0.90 (± 0.18)	8.26	0.7	1.60
2500	3243	- 1.26 (± 0.21)	8.91	0.0	1.60
2500	3245	- 1.38 (± 0.23)	7.99	0.7	0.80
2500	3250	- 0.37 (± 0.49)	7.27	6.7	1.07
5000	3238	not analyzable due to artifacts			
5000	3241[d]	- 0.17 (± 0.33)	7.10	2.7	1.73
5000	3267	- 0.86 (± 0.33)	9.01	2.7	2.93
DMN	3249	19.05 (± 1.76)	6.56	98.0	1.47
DMN	3266	17.47 (± 1.78)	6.65	98.0	1.33
DMN	3270	16.83 (± 4.00)	5.74	97.3	0.53

[a] UDS = Mean net nuclear grain count from triplicate coverslips (150 total cells)
[b] Triplicate coverslips (150 total cells)
[c] Percent of S-phase nuclei on triplicate coverslips (1500 total cells scored)
[d] UDS = Mean net nuclear grain count from duplicate coverslips (150 total cells for UDS and cytoplasmic grains; 1500 cells for S-phase)
VC = Vehicle Control, 5 mL/kg of corn oil
DMN = Positive control dimethylnitrosamine, 10 mg/kg

SECTION 7

1,3-DICHLOROTETRAFLUOROBENZENE GENOTOXICITY SUMMARY EVALUATION

Myhr, B[a]

1,3-Dichlorotetrafluorobenzene (DCFB), a fully substituted halogenated benzene, was tested for potential genotoxic activity by application to several *in vitro* assays, as described below, and an *in vivo* assay for DNA damage. Because the substance has little or no solubility in aqueous medium, the polyalcohol Pluronic® F68 was used as an emulsifying agent for the *in vitro* assays. After homogenization in 10% w/v Pluronic® F68, a stable suspension of tiny droplets was obtained that did not coalesce when diluted into culture media at Pluronic® F68 concentrations of 0.8-1% w/v. Glass containers and pipets were used for DCFB emulsions and treated cultures because the DCFB emulsions were found to score and soften polystyrene surfaces. For the *in vivo* study, DCFB was solubilized in corn oil and administered by oral gavage.

The Ames Salmonella reverse mutation assay was performed with the preincubation methodology in order to maximize contact between the bacteria and DCFB emulsions. Strains TA98, TA100, TA1535, TA1537 and TA1538 were used in the presence and absence of a rat liver S9 metabolic activation system (Aroclor 1254-induced). DCFB was somewhat more toxic without S9, but toxicity was clearly evident at 1000 µg/plate under both test conditions. Dose ranges of 1 to 2500 µg/plate without S9 and 5 to 5000 µg/plate were assayed for the induction of revertants. No increases in revertants were obtained, so no evidence for mutagenic activity by DCFB was collected.

In mammalian cell culture, DCFB was tested for mutagenic activity at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary (CHO) cells. After a four hour exposure in the presence and absence of the rat liver S9 activation system, excessive toxicity was obtained at 100 µg/mL in a dose rangefinding study. A mutation assay performed without S9 over a DCFB concentration range of 5 to 100 µg/mL yielded a survival range of 98 to 22.7% and no significant increases in mutant frequency. Similarly, a mutation assay with S9 over a dose range of 10 to 250 µg/mL yielded a survival range of 107 to 2.8% and no increase in mutant frequency. Therefore, DCFB was toxic to CHO cells without causing any detectable mutagenesis at the HGPRT locus.

CHO cells were also examined for cytogenetic damage in assays for DCFB-induced chromosomal aberrations and sister chromatid exchanges (SCEs). Lethality was observed at

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167 µg/mL with or without S9, and a substantial delay in cell cycle was observed at 50.1 µg/mL in the absence of S9 but not with S9. Accordingly, a dose range of 25 to 100 µg/mL was analyzed without S9, using a delayed harvest of 20 h. No increases in chromosomal aberrations were observed. With two-hour treatments with S9, 150 µg/mL of DCFB was extremely toxic, and no increases in aberrations were found over an analyzed dose range of 15 to 150 µg/mL, using a standard 10 h harvest. The assay for SCE showed a weak response only at 50.1 µg/mL, with and without S9, that was not confirmed in repeat trials that included higher doses. Thus, DCFB did not interact with cultured CHO cells in such a way as to induce chromosomal aberrations or cause any consistent genetic changes as indicated by SCE.

DCFB was assayed for its ability to induce morphological transformation in vitro in cultures of mouse BALB/c-3T3 cells, both in the presence and absence of a rat liver S9 metabolic activation system. The treatment periods were two hours with S9 and 72 h without S9. The two test conditions resulted in similar toxicities, as measured by the clonal survivals of ouabain-resistant cells in the presence of the wildtype monolayer cultures. A survival range of 105 to 9% was obtained for a DCFB concentration range of 25 to 250 µg/mL. The number of transformed foci was not increased by any of the treatments, so DCFB had no detectable activity as a transforming agent to BALB/c-3T3 cells.

An *in vivo* assessment of genetic activity was performed by dosing male F-344 rats with four doses of DCFB ranging from 625 mg/kg to the maximum amount 5000 mg/kg. Approximately four hours after single oral administrations, primary hepatocyte cultures were established to determine the degree of DNA repair (unscheduled DNA synthesis (UDS)) by labeling with ^{3}H-thymidine. An autoradiographic analysis of the extent of nuclear labeling and percent of cells in DNA repair showed no increases over cultures from control rats. Therefore, no evidence was obtained for DNA damage in the liver caused by the DCFB treatments.

The results of the above genetic tests indicate the DCFB does not interact with genetic material. No responses were observed for any of the genetic endpoints tested, using DCFB treatments that included highly toxic treatments or an excessive dose of 5000 mg/kg in the case of the rat liver UDS study. This lack of activity in the genetic test battery would predict essentially no genetic risk from exposures to DCFB.

SECTION 8

APPENDIX A

SUMMARY OF TESTING ON 1,3-DICHLOROTETRAFLUOROBENZENE WITH RAT PRIMARY HEPATOCYTES

Cifone, M.A[a]

ABSTRACT

An attempt was made to evaluate 1,3-dichlorotetrafluorobenzene (DCFB) in the *In Vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis (UDS) Assay. The test material required that testing be performed in closed containers. Several containers and conditions were tried and none resulted in acceptable levels of survival. While conditions for the UDS assay were being developed, an attempt was made to obtain toxicity information. Four cytotoxicity assays were initiated but the data from the first three studies were unacceptable. The fourth study demonstrated that the test material was excessively toxic to rat primary hepatocytes at concentrations at and above 50.2 µg/mL. High toxicities were observed at 10.0 and 1.00 µg/mL. An attempt was made to perform the UDS assay. Hepatocytes from two rats were exposed to DCFB at concentrations from 1.00 to 0.005 µg/mL. Erratic toxicity curves were observed and the cells had morphologies that were unacceptable for analysis. The studies were terminated because acceptable conditions for performing the UDS assay would require further developmental studies.

INTRODUCTION

Fresh hepatocytes obtained from rat liver will attach to a surface in culture and continue to metabolize for several days without undergoing cell division. Only a small percentage of the cells enter S-phase (replicative DNA synthesis). Therefore, if ^{3}H-thymidine (^{3}H-Tdr) is introduced in the culture medium, little or no label will be incorporated into nuclear DNA. The addition of a test material that interacts with the DNA often stimulates a repair response in which the altered portion of DNA is excised and the missing region replaced by DNA synthesis. This synthesis of DNA by nondividing cells is known as unscheduled DNA synthesis (UDS) and can be measured by determining the amount of ^{3}H-Tdr incorporation using an autoradiographic method. Cells involved in DNA replication are recognized by heavy labeling of the nuclei and are excluded from the evaluation of UDS activity. Autoradiographic measurement of DNA repair is highly sensitive and appears to correlate very well with the known mutagenic or carcinogenic activities of chemicals (Williams, 1977).

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Furthermore, the use of primary hepatocytes has the advantage that these cells have sufficient metabolic activity to eliminate the need for the addition of a microsomal activation system.

The objective of this assay was to detect DNA damage caused by the test material, or an active metabolite, by measuring UDS in rat primary hepatocytes *in vitro*. The existence and degree of DNA damage was inferred from an increase in net nuclear grain counts in treated hepatocytes when compared to untreated hepatocytes. The types of DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including ^{3}H-Tdr) into DNA.

MATERIALS AND METHODS

Test Material: 1,3-Dichlorotetrafluorobenzene, 99%, Lot No. 01609LV
Physical Description: Clear, colorless liquid

Indicator Cells

The indicator cells for these assays were hepatocytes obtained from adult male Fischer 344 rats (150-300g), purchased from Charles River Breeding Laboratories, Incorporated. The animals scheduled for this assay were fed Purina Certified Rodent Chow (Formula 5002) and water *ad libitum*. Two animals, identified by cage card, were used for each trial of the UDS assay after a minimum quarantine period of five days. One rat was used for each cytotoxicity assay.

The cells were obtained by perfusion of the liver *in situ* with a collagenase solution. Monolayer cultures were established on plastic coverslips in culture dishes and were used the same day for initiation of the UDS assay. All cultures were maintained as monolayers at about 37°C in a humidified atmosphere containing approximately 5% CO_2.

Medium

The cell cultures were established in Williams' Medium E supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 µg/mL streptomycin sulfate, and 150 µg/mL gentamicin (WME +). In some cases 25 mM Hepes buffer (pH 7.2) was included in the medium. After the establishment period, the serum was removed. This latter culture medium is referred to simply as WME1. This also contained Hepes buffer. If Hepes buffer was not added, the cultures were blanketed with 5% CO_2 to maintain the correct pH range

Controls

Negative Control

A negative control consisting of assay procedures performed on cells exposed only to the test material solvent was performed. The solvent for the UDS assay was deionized water containing 1.0% F-68 Pluronic®. The final concentration of solvent in the medium was 10%. An untreated control consisting of assay procedures performed on mock-exposed cells was also included.

Positive Control

The positive control compound is known to induce UDS in rat hepatocyte primary cell cultures. 2-acetylaminofluorene (2-AAF) at 4.4×10^{-7} M (0.10 µg/mL) was used as the positive control.

EXPERIMENTAL DESIGN

Dosing Procedure

The test material was dissolved at the highest desired concentration in deionized water containing 10% F-68 Pluronic®. 1,3-Dichlorotetrafluorobenzene (DCFB) was immiscible with the solvent and was dispersed with a Tissumizer® to obtain a milky white suspension. Lower concentrations were then prepared by dilution with 10% F-68 Pluronic® (initial studies) or 1% F-68 Pluronic® (later studies including UDS assay). The dosing treatments were prepared by performing 1:10 dilutions of the stocks into WMEI containing ^{3}H-Tdr (final concentration, 5 µCi/mL).

Fresh preparations of test material in the vehicle were used for the biological testing. Treatments were initiated by replacing the medium on the cell cultures with WMEI containing the test material at the desired concentrations and 5 µCi/mL ^{3}H-Tdr (20 Ci/mM).

Dose Selection

Preliminary cytotoxicity assays were performed to determine the doses to be initiated in the UDS assay. The initial cytotoxicity assay was initiated with treatments from about 5000 to about 50.0 µg/mL. The concentrations tested were lowered when it was observed that the test material was highly toxic. Three cytotoxicity assays were performed.

UDS and Cytotoxicity Assays

This assay was based on the procedures described by Williams (1977, 1980). The hepatocytes were obtained by perfusion of livers *in situ* for about four minutes with Hanks' balanced salts (*Ca^{++} and Mg^{++} free*) containing 0.5 mM ethyleneglycol-bis (-aminoethyl ether)-N, N-tetraacetic acid (EGTA), and HEPES buffer at pH 7.2. Then WMEI containing 50-100 units/mL of collagenase was

perfused through the liver for about 10 min. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WMEI culture medium and collagenase. Clumps of cellular tissue and debris were removed by allowing the clumps to settle to the bottom of the plate. The supernatant was centrifuged and the cell pellet resuspended in WME +. After obtaining a viable cell count, a series of 8 dram Shell vials (some containing a 15-mm round, plastic coverslip) was inoculated with viable cells in WME +.

An attachment period of 1.5 to 2 h at approximately 37°C in a humidified atmosphere containing about 5% CO_2 was used to establish the cell cultures. Unattached cells were then removed and the cultures were refed with WMEI. The assays were initiated within three hours by replacing the media in the vials with WMEI containing 5 µCi/mL ^{3}H-Tdr, (20 Ci/mM) and the test material at the desired concentration. Cytotoxicity assays were initiated without the added label. After treatment for 18 to 24 h, the assays were terminated by washing the cell monolayers twice with WMEI. For the cytotoxicity assays, two cultures were used to monitor toxicity. For the UDS assay, three of the cultures from each treatment were washed with WMEI containing one mM thymidine and were further processed as described below. Another two cultures used to monitor the toxicity of each treatment were refed with WMEI and returned to the incubator. At 20 to 24 h after the initiation of the treatments, viable cell counts (trypan blue exclusion) were determined to estimate cell survival relative to the negative control.

The nuclei in the labeled cells were swollen by addition of 1% sodium citrate to the coverslips for 8 to 10 min, and then the cells were fixed in acetic acid:ethanol (1:3) and dried for at least 24 h. The coverslips were mounted on glass slides, dipped in an emulsion of Kodak NTB2, and dried. The coated slides were stored for 7 to 10 days at 4°C in light-tight boxes containing packets of Drierite. The emulsions were then developed in D19, fixed, and stained using Williams' modified hematoxylin and eosin procedure.

The cells were examined microscopically at approximately 1500 × magnification under oil immersion and the field was displayed on the video screen of an automatic counter. UDS could not be measured because of the morphological appearance of the cells.

RESULTS AND DISCUSSION

The test material, DCFB, was insoluble in medium and formed large globules that settled to the bottom of the vessel. In order to disperse the test material in the solvent and allow even exposure of the target hepatocyte cells, 10% F-68 (w/v in water) was used to suspend the test material. DCFB was mixed with 10% F-68 Pluronic® and the test material was dispersed using a Tissumizer®. A milky white suspension formed that appeared to be stable. The highest concentration prepared was 50 mg/mL and, in most cases, this suspension was diluted with 10% F-68 Pluronic® to obtain a series of

stock solutions. In some of the later experiments, the 10% F-68 Pluronic® was reduced to 1% (for diluting the stocks) in order to reduce any possible effects of the F-68 Pluronic® on the hepatocytes. The final dosing concentrations were prepared by performing 1:10 dilutions of the F-68 Pluronic® stocks with WME medium containing ^{3}H-Tdr (final concentration, 5 µCi/mL). No labeled thymidine was added to the treatments used for cytotoxicity tests. The test material formed a good suspension in the medium.

The cytotoxicity and UDS assays were initiated by replacing the medium on the cells with medium containing the appropriate concentration of test material (and ^{3}H-Tdr, if appropriate).

Because of the volatile nature of DCFB, and in order to maintain a stable and representative concentration of the test material during the dosing period, it was necessary to perform the assays in closed containers. The hepatocytes were seeded into eight dram glass Shell vials containing 15-mm polyester coverslips instead of the usual 35-mm plastic culture dishes containing 25-mm round polyester coverslips. In the early experiments, 25 mM Hepes buffer was included in the medium to maintain the appropriate pH. It was later found to be slightly toxic and the cultures were blanketed with 5% CO_2, which also maintained the appropriate pH. The use of closed containers proved to be a continual problem throughout the studies. A brief description of studies performed to alleviate this problem is shown in the Appendix B.

To select the appropriate doses for the UDS assay, it was decided that a preliminary cytotoxicity assay would be performed. This is not usually necessary for this assay but was instituted because of the special conditions and the request for a two-rat study. Four cytotoxicity assays were initiated. Trials 1 and 2 were initiated with treatments from about 5000 to about 50.0 µg/mL. Both assays were terminated because all the cells were dead, even the solvent control cells. The controls in Trial 3 were somewhat better, but still not acceptable. Trial 4 (Table 8-A-1) was initiated at concentrations from 1000 to 1.00 µg/mL and the test material was highly toxic at 10.0 and 1.00 µg/mL. Higher concentrations were lethal.

The UDS assay was initiated with cells from two rats at concentrations from 1.00 to 0.005 µg/mL. The cytotoxicity curve was erratic in both studies and the cells were unacceptable for analysis. The assay was terminated.

REFERENCES

Williams, G.M. 1977. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture. *Cancer Res.*, 37:1845-1851.

Williams, G.M. 1980. The detection of chemical mutagens-carcinogens by DNA repair and mutagenesis in liver cultures. In: F. de Serres and A. Hollaender, (eds.) *Chemical Mutagens*, Vol. 6, Plenum Press, NY, pp. 61-79.

TABLE 8-A-1. SUMMARY OF DATA FROM TRIAL 5 OF THE RAT HEPATOCYTE CYTOTOXICITY ASSAY USING 1,3-DICHLOROTETRAFLUOROBENZENE

Test Condition	Concentration	Survival at 21.9 hrs (%)***
Negative Control Media	------	122.4
Solvent Control 1% F-68	10%	100.0
Test Material: (µg/mL)		
	1000	0
	502	0
	100	0
	50.2	0
	10.0	26.9
	1.0	55.3

***Survival = Number of viable cells per unit area relative to the solvent control

APPENDIX B

SUMMARY OF ATTEMPTS TO PERFORM UDS ASSAY IN CLOSED CONTAINERS

Three UDS studies were requested that required the assays be performed in closed glass containers. This is difficult in the UDS assay because hepatocytes do not attach well to glass and do not survive well in closed containers The requirement for glass coverslips was waived when the polyester coverslips we routinely use were shown to be resistant to the chemicals. However, it was still a requirement that closed containers be used. A preliminary experiment was performed to determine the technical problems associated with this requirement.

In the preliminary experiment, several glass containers containing coverslips were seeded with cells; Hepes buffer was added to some of the cultures to control the pH. The cells attached very well after 1.5 to 2 h and the toxicity tests were initiated using Shell vials. When the toxicity tests were performed, no cells were alive 18 to 20 h later. It appeared that cell attachment was normal but long term survival was a problem. Several experiments were then performed to determine the parameters that affected survival. The following changes were made:

- Hepes buffer was not used and the cultures were gassed with 5% CO_2 to maintain an acceptable pH.
- The culture vessels were pretreated with medium containing 20% serum.
- The number of cells seeded was reduced.
- The volume of medium used was increased.
- Other culture vessels were tried again but rejected because they were not currently available or because of technical reasons.
- The dosing and feeding procedures were changed to make the cell handling more gentle.

Cytotoxicity studies were initiated again but were only partially successful. Additional studies were performed and the following changes were made:

- 1% serum was added to the dosing medium
- At least twice the number of cultures was set up
- The requirement for 50% survival of the cells in the control cultures was waived.

The cytotoxicity tests were again initiated and were partially successful and doses could be chosen The UDS assays were initiated

In the UDS assays initiated, the results were variable In some cases, cells appeared to survive but the toxicities were erratic Slides were prepared and autoradiography was performed When the

slides were developed, UDS could not be determined because most of the cells were rounded and cell labeling was abnormal. In addition, all of the positive control cultures were excessively toxic. None of the changes that were instituted avoided the problem of random loss of cultures or whole dose groups. It appeared that the cells were more fragile under conditions where closed containers were used and the parameters involved were not readily controlled. Reliable results could not be obtained from the cells under the conditions requested.

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